University of Alberta

Delayed Wallerian degeneration of sympathetic axons: A potential role of NAD+ and the MEK/Erk pathway

by

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Abstract

Axon death is an important feature in development of both the nervous system and in a variety of neuron degenerative diseases. Until recently, death of an axon was thought to occur passively, but this view changed with the discovery of a naturally occurring mutation in mice called Wallerian degeneration slow (Wlds) (Lunn et al., 1989). Wlds-mediated delays in degeneration were associated with the expression of a fusion protein that expressed an incomplete E4 ligase and a functional nicotinamide mononucleotide adenylytransferase-1 (Nmnat-1). Delays in axonal degeneration were originally thought to be caused by the incomplete E4 ligase, but recent studies have shifted focus onto the role of Nmnat-1. Nmnat-1 is responsible for the formation of NAD, and this body of work focused on the effects of exogenous application of NAD to transected axons. NAD applied locally to the axon resulted in delayed Wallerian degeneration, and this protection was shown to be dependent on the MEK-Erk pathway. NAD application maintained Erk phosphorylation for 24 hours, and Erk1/2 inhibition resulted in normal degeneration of NAD-treated, transected axons. Also, treatment of transected axons with the drugs; sirtinol, an inhibitor of sirtuins (which uses NAD as a substrate), and Ro-31-8220, an inhibitor of p90Rsk (a downstream target of Erk1/2), increased metabolic activity of transected axons given NAD. This work suggests the MEK/Erk1/2 pathway is important in survival of transected axons and activity-dependent survival may have therapeutic implications for neurodegenerative diseases and neural trauma.

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Dedication

I dedicate this work to Meaghan. You are my sanity.

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List of Abbreviations

Aβ	amyloid beta
ALS	amyotrophic lateral sclerosis
ATP	adenosine triphosphate
BAD	Bcl-2 associated death protein
Bcl-2	B-cell leukemia/lymphoma 2
Bcl-Xl	Bcl-Xl protein
BSA	bovine serum albumin
CB/PAx	Cell bodies/proximal axons
CNS	central nervous system
DAx	Distal axons
DMSO	dimethylsulfoxide
Erk1/2	Extracellular signal-regulated kinase1/2
GSK-3	glycogen synthase kinase-3
HPLC	high performance liquid chormatography
JNK	c-Jun N-terminal kinase
LDH	lactose dehydrogenase
L-15	Leibovitz's medium
MCL-1	myeloid cell leukemia sequence-1
MEK1/2	Mitogen-activated protein kinase Erk1/2
MSK1/2	mitogen and stress-activated protein kinases 1/2
Na	nicotinate
NaAD	nicotinate adenine dinucleotide
NAD	nicotinamide adenine dinucleotide
Nam	nicotinamide
NaMN	nicotinate mononucleotide
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NMN	nicotinamide mononucleotide
Nmnat1	Nicotinamide mononucleotide adenyltransferase-1
PAGE	polyacrylamide gel electrophoresis
PARP	poly ADP-ribose polymerase
PBS	phosphate buffer saline
PKC	protein kinase C
PNS	peripheral nervous system
PVDF	polyvinylidene fluoride
p90Rsk	p90 ribosomal S6 kinase
Rbp7	retinol binding protein 7
RT-PCR	reverse transcription – polymerase chain reaction
SDS	sodium dodecyl sulphate
SIRT	sirtuin
Sir2	silent information regulator 2 protein
TBS	tris buffer saline
TBS-T	tris buffer salin + 0.1% tween20
VEGF	vascular endothelial growth factor

UPS	ubiquitin-proteasome system
Wlds	Wallerian degeneration slow

1. Introduction

1.1. The importance of axonal degeneration:

One of the earliest examples of axonal death in the nervous system is the loss of excess or inappropriately wired axon connections that are made during development. This occurs through a process known as 'pruning' or 'branch elimination' and is important for the 'correct' wiring of the nervous system (reviewed by (Zhai et al., 2003, Low and Cheng, 2005)). Axonal death also occurs in neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease, and often precedes cell body death in unhealthy neurons (reviewed by (Cavanagh, 1979)). Many treatments for neurological disorders, including glutamate receptor inhibition to treat Alzheimer's disease (reviewed by (Zimmermann et al., 2005)) and vascular endothelial growth factor (VEGF) application to treat amyotrophic lateral sclerosis (ALS) (reviewed by (Lambrechts et al., 2004, Storkebaum et al., 2004)), target the neuron as a whole rather than specifically targeting the axon. Neuronal cell death may occur at or near the end of disease progression, as neuronal protection fails to prevent development of symptoms. For example, anti-apoptotic proteins, including Bcl-2 (B-cell leukemia/lymphoma 2) and Bcl-xL, inhibit caspase-dependent cell death pathways by preventing cytochrome c release and have been shown to prevent apoptosis in many cell types (reviewed by (Chao and Korsmeyer, 1998, Zhai et al., 2003)). When Bcl-2 is overexpressed in a mouse model of motor neuron degeneration, cell bodies of motor neurons were protected from apoptosis, but axonal degeneration and disease progression still occurred (Sagot et al., 1995). The vast majority of the nervous system is interconnected by axons and dendrites, neuronal components, that send signals to and from neurons and/or the cells they

innervate. If axons can still die after preventing neuronal apoptosis, understanding mechanisms involved in axonal death is crucial to maintaining axon function. This understanding of axonal death will be beneficial in deriving better therapeutic treatments of various neurodegenerative diseases and traumas to maintain neuronal function.

1.2. Types of Axonal Degeneration:

One model for studying mechanisms of axon degeneration is Wallerian degeneration. Wallerian degeneration occurs when an axon is cut and the portion of axon distal to the cut disintegrates (reviewed by (Coleman and Perry, 2002, Raff et al., 2002)). This characteristic breakdown of the axon results in a destabilization and disintegration of the axonal cytoskeleton and breakdown of the axonal membrane (reviewed by (Ehlers, 2004)). Wallerian degeneration can occur in central and peripheral nervous systems (CNS and PNS) and can be caused by trauma and ischemia that cause damage to the axon (Buss et al., 2004, Thomalla et al., 2005). Also of importance, axons that undergo Wallerian degeneration do not undergo caspase activation (Finn et al., 2000), a hallmark of programmed cell death or apoptosis, which suggests that different mechanisms of cellular death are involved.

A more common form of axonal degeneration is seen in a variety of neurodegenerative diseases and is referred to as 'dying back' axon degeneration. In dying back axon degeneration, the axon degeneration progresses from the distal end of the axon towards the neuronal cell body (reviewed by (Coleman and Perry, 2002, Raff et al., 2002)). Dying back axonal degeneration occurs over a period of weeks to months and is responsible for the development of symptoms as axons detach from the targets they

innervate, resulting in functional losses. Although Wallerian degeneration and 'dying back' degeneration are considered to be distinct from one another, they may share similar mechanisms.

1.3. The Wlds mutation, from passive to active degeneration:

Classically and logically, it has been thought that transport of materials from the neuronal cell body to the axon was needed to maintain axon structure and function. An axon that has been separated from its neuronal cell body no longer receives transported material from the cell body; this loss of transport of materials was thought to result in the rapid breakdown of axons as they could no longer replenish the much needed proteins and biosynthetic materials provided by the cell body. The view of axon degeneration as a passive process has changed dramatically with the discovery of a naturally occurring, spontaneous mutation in mice termed Wallerian degeneration slow (Wlds) (Lunn et al., 1989). In mice expressing the Wlds protein it was found that axons separated from their cell bodies were able to survive for weeks *in vivo* and days *in vitro*. This Wlds-mediated survival compared to a lifespan of only days *in vivo* and hours *in vitro* in wildtype mouse axons. In addition to delaying Wallerian degeneration, Wlds protein expression protects axons of intact neurons from a variety of insults, including trophic factor withdrawal (Deckwerth and Johnson, 1994) and vincristine toxicity (Wang et al., 2001a). These results further suggest there may be a general regulatory mechanism or mechanisms mediating axonal degeneration rather than a loss of biosynthetic materials from the cell body.

The Wlds phenomenon has been attributed to a tandem triplication of 85kb (Coleman et al., 1998) that occurs in the distal arm of chromosome 4 (Lyon et al., 1993). The 85kb region has been mapped and determined to wholly or partially encode genes for 3 proteins: Ube4b(E4) ubiquitin ligase, nicotinamide mononucleotide adenylyltransferase1 (Nmnat1) responsible for the formation of nicotinamide adenine dinucleotide (NAD) (Magni et al., 1999), and retinol binding protein 7 (Rbp7) (Conforti et al., 2000). Triplication of the chromosomal fragment encoding these 3 genes results in the formation of the chimeric Ube4b/Nmnat1 protein and the overexpression of Rbp7 (Conforti et al., 2000). Although Rbp7 is overexpressed in the Wlds mouse as determined by western blot analysis, no Rbp7 mRNA could be detected by Northern blot in the brains of Wlds and wildtype mice (Conforti et al., 2000). However, semi-quantitative RT-PCR did detect an increase in expression of Rbp7 in Wlds mice brains compared to wild type (Conforti et al., 2000). The authors of this study suggest Rbp7 is an unlikely source of the Wlds phenomenon due to the low Rbp7 mRNA and protein expression in the brain, but it has not been completely excluded. Instead, research has focused on the role of Ube4b/Nmnat1 chimera in axon protection.

Transgenic mice expressing increasing levels of Ube4b/Nmnat1 chimeric protein displayed increased axonal protection. In fact, transgenic mice expressing levels of Ube4b/Nmnat1 protein similar to Wlds mice also had similar degrees of axonal protection (Mack et al., 2001) suggesting Ube4b/Nmnat1 is responsible for the Wlds phenomenon. This chimeric protein is now referred to as the Wlds protein and consists of 70 amino acids of an E4 ubiquitin ligase and a fully functional Nmnat1. This raises

the question of which portion of this chimeric protein (or both) causes delays in axonal degeneration.

1.4. Ubiquitin-Proteasome system vs. Nmnat1 as a mediator of delayed axon degeneration:

Originally, the incomplete E4 ubiquitin ligase of the Wlds protein was thought to be responsible for the delay in axon degeneration through affecting trafficking of proteins to the 26S proteasome. The 26S proteasome is responsible for proteolytically degrading ubiquitinated proteins, so affecting protein trafficking to the proteasome could potentially delay general protein breakdown. Ubiquitination is a process by which proteins are modified by the addition of a 76 amino acid polypeptide, ubiquitin. This addition of ubiquitin to a substrate occurs via a three step enzymatic process. Ubiquitin is activated in an ATP-dependent manner by an E1 enzyme that forms a high energy ubiquitin-E1 intermediate. E1 is then replaced by an E2 conjugating enzyme allowing for an interaction between E2 conjugating enzyme and an E3 ligase bound to the substrate to be ubiquitinated. The E3 ligase then transfers the ubiquitin to the target substrate (reviewed by (Roos-Mattjus and Sistonen, 2004)). Ubiquitin is attached to lysine residues on target proteins and can be in either a monomeric form or in chains of ubiquitin, which affect the fate of the target protein (reviewed by (Haglund and Dikic, 2005)). Formation of poly-ubiquitin chains on protein substrates targets these proteins to the 26S proteasome-protease complex resulting in their degradation (reviewed by (Roos-Mattjus and Sistonen, 2004)). E4 ligases, such as the Ube4b of Wlds, were originally thought to be a new group of ubiquitin ligases, as they contain a novel U-box domain. Recently E4

ligases have been reclassified as a third family of E3 ligases, in addition to HECT and RING-finger E3 ligases.

In support of the idea that the ubiquitin-proteasome system (UPS) is involved in axon degeneration, proteasome inhibition by pharmacological and genetic means caused delays in degeneration of transected axons and trophic factor-deprived axons (Zhai et al., 2003). The role of the UPS in axon degeneration may be affected by the incomplete expression of the E4 ligase. This incomplete protein may affect ubiquitin signaling to the proteasome by altering ubiquitin chain formation, and could cause the delays in axon degeneration seen in Wlds expressing animals. MacInnis and Campenot (2005) also demonstrated that proteasome inhibitors could delay axon degeneration of both transected and nerve growth factor (NGF)-deprived axons. Treatment with proteasome inhibitors resulted in a general maintenance of the axon, while pharmacological inhibition of Erk1/2 activity prevented the protection of NGF-deprived or transected axons by proteasome inhibitors. These results suggest that although proteasome inhibition can prevent transected and trophic factor-deprived axons from dying, Erk1/2 activity is required for their survival.

Although involvement of the proteasome in axon degeneration may still provide therapeutic targets for treatment of neurological disorders, recent studies have focused on the role of Nmnat1 in delayed axon degeneration seen with Wlds expression. Studies using viral expression vectors overexpressing various portions of the Wlds protein, either the Ube4b moiety and/or the Nmnat1 moiety, have illustrated that delays in axon degeneration occur only when functional Nmnat1 is expressed in the neuron (Araki et al., 2004). Nmnat1 appears to mediate its effects by production of NAD, as exogenous

application of NAD also resulted in delays in axonal degeneration in dorsal root ganglia (Araki et al., 2004, Wang et al., 2005). There is dispute as to whether NAD mediates its protective effects in the cell body through downstream effectors that are either transported to the axon or signal to downstream effectors present in the axon, or if NAD directly affects the axon. This conflict is in part due to the lack of chemical separation in mass culture conditions, so both cell bodies and axons are equally exposed to treatments. Also, Nmnat1 is thought to function primarily in the nucleus (Magni et al., 2004b). Work by Wang et al., (Wang et al., 2001b) demonstrated that overexpression of Wlds and Nmnat1 proteins resulted in Nmnat1 localization in the axons of dorsal root ganglion neurons. Given that axon separation from the cell body initiates Wallerian degeneration, this implies that whatever protects the axon from degenerating must be present in the axon. The simplest explanation is that Wlds or Nmnat1 proteins mediate their protective effects locally in the axons through the production of NAD, but it is possible that downstream effectors of nuclear Wlds or Nmnat1 may be transported into the axon.

1.5. NAD function in the cell:

NAD is a co-enzyme responsible for electron transfer in metabolic pathways and also functions as a substrate for certain enzymes. NAD is formed by two pathways, termed the *de novo* and salvage pathways. The *de novo* pathway results in the formation of NAD from the amino acid tryptophan while the salvage pathway reconstructs NAD from breakdown products of NAD-dependent enzymatic processes (Magni et al., 2004a). These two processes are not completely separate from one another as the *de novo*

pathway feeds into the salvage pathway, in which Nmnat1 functions to convert nicotinamide mononucleotide to NAD (Figure 1.1).

Many NAD-dependent proteins are located in the nucleus, including poly ADP-ribose polymerase (PARP), and members of the sirtuin (SIRT) family (Michishita et al., 2005). Nmnat1 has also been localized to the nucleus (Magni et al., 2004b) and is part of the salvage pathway responsible for reforming NAD. Nmnat1 maintains nuclear NAD levels and provides NAD for proteins such as PARP and the SIRTs to exert their effects.

The sirtuins (SIRT) are a family of NAD-dependent proteins and are homologs to silent information regulator 2 (Sir2) in yeast, which have been implicated in DNA repair (Smith and Boeke, 1997) and longevity of the organism (Kaeberlein et al., 1999, Tissenbaum and Guarente, 2001). Of the seven mammalian SIRTs, SIRT1 has been implicated in causing delayed axonal degeneration in Wlds-expressing dorsal root ganglia neurons and with exogenous application of NAD to dorsal root ganglia neurons (Araki et al., 2004). Araki et al., (Araki et al., 2004) demonstrated the importance of SIRT1 activity in NAD-mediated delayed axon degeneration by inhibiting SIRT1 activity both pharmacologically and genetically. SIRT1 inhibition resulted in normal, rather than delayed, degeneration after transection of NAD pretreated neurons.

The role of SIRT1 in Wlds protection has recently been challenged by Wang et al., (Wang et al., 2005), who show that SIRT1 is not involved in Wlds delays in axon degeneration. Nmnat1 and NAD-mediated protection of transected axons is unaffected after genetically knocking out SIRT1 or pharmacologically inhibiting its activity in dorsal root ganglia neurons. Wang et al., (Wang et al., 2005) also show by high performance liquid chromatography that overexpression of Wlds maintained NAD levels in axons

after injury and the sustained NAD levels in Wlds-expressing axons protect them from degeneration by maintaining energy production in the axon. NAD is needed in glycolysis to form pyruvate, which then enters the mitochondria and drives energy production. Exogenous pyruvate was applied to transected dorsal root ganglia neurons and it had similar protective effects on the axons as exogenous NAD application. Although NAD-dependent SIRT1 may have protective functions, the delays in axon degeneration after Wlds expression, Nmnat1 expression and exogenous NAD application suggest that Wlds, Nmnat1 and NAD function in axons to protect them from degeneration.

NAD-dependent enzymes are also present in the cytosol, including the protein deacetylases SIRT2 (North et al., 2003) and SIRT3 (Onyango et al., 2002). The NAD salvage pathway may be important in the cytosol given the reported decreases in the levels of NAD by HPLC analysis of transected axons of dorsal root ganglia neurons (Wang et al., 2005). The salvage pathway allows for the restoration of NAD levels in the cytosol to maintain the NAD:NADH ratio as well as to allow NAD-dependent proteins in the cytosol such as the tubulin deacetylase, SIRT2, to function (North et al., 2003).

1.6. Erk1/2 activity as a general mechanism of axon protection:

Extracellular signal related kinase 1/2 (Erk1/2) is one of the main signaling molecules in the Erk signaling cascade. Erk1/2 activity regulates a variety of cellular processes including proliferation, differentiation and cell cycling (reviewed by (Yoon and Seger, 2006)). The main molecules in the Erk signaling pathway are the kinases, Raf, mitogen-activated protein kinase Erk1/2 (MEK1/2), and Erk1/2. Activation of Raf results in a phosphorylation cascade where Raf phosphorylates MEK1/2 and MEK1/2 in

turn phosphorylates Erk1/2 (Figure 1.2). Erk1/2 further phosphorylates downstream targets to initiate various cellular processes.

Erk1/2 activity has been shown to be both beneficial and detrimental to neurons (reviewed by (Hetman et al., 2002, Chu et al., 2004)), but the exact cellular mechanisms of Erk1/2 activity in death and survival remain unclear. An example of Erk1/2 activity negatively affecting neuronal survival is Erk1/2-mediated overactivation of PARP. PARP is activated by DNA damage and adds ADP-ribose units to various proteins to recruit them for DNA repair. PARP breaks down NAD to obtain the ADP-ribose units. Overactivation of PARP by Erk1/2 in DNA damage-induced stress leads to energetic deprivation due to a decrease in NAD levels and results in necrosis in SH-SY5Y neuroblastoma cell lines (Arai et al., 2004). Cell death was reduced in these cells with pharmacological inhibition of Erk1/2 (Arai et al., 2004) further suggesting a role for Erk1/2 activity in neuronal death.

Despite these examples of Erk1/2 activity in neuronal death, Erk1/2 activity has also been implicated in neuronal protection. Activation of the Erk1/2 pathway by transforming growth factor β-1 (TGF-β1) of hippocampal neurons maintained Bcl-2 associated death (BAD) protein phosphorylation and protected neurons from staurosporine-induce apoptosis (Zhu et al., 2002). Also, androgen application mediated an increase in Erk1/2 phosphorylation in hippocampal neurons (Nguyen et al., 2005). These increases in Erk1/2 phosphorylation correlated with increases in BAD phosphorylation, preventing its activation and protected hippocampal neurons from amyloid beta (Aβ) toxicity (Nguyen et al., 2005). Pharmacological inhibition of Erk1/2 also decreased BAD phosphorylation levels and caused Aβ-induced death (Nguyen et al.,

2005). In a recent study, Erk1/2 activity was shown to be necessary for maintaining axon function of both transected and trophic factor-deprived axons when axon degeneration was prevented by proteasome inhibition (MacInnis and Campenot, 2005). As discussed earlier, these results demonstrated that proteasome inhibitors delayed axon degeneration and maintained cellular functions. This delay in axon degeneration was dependent upon Erk1/2 activity as inhibition of Erk1/2 phosphorylation restored degeneration in the presence of the proteasome inhibitor, MG132.

Previous studies have implicated Erk1/2 activity in a variety of neuroprotective phenomena after injury. Exogenous N-methyl-D-aspartate (NMDA)-mediated excitotoxic damage of hippocampal slice cultures results in decreases in synaptic activity (Ferchmin et al., 2003). These decreases in activity are prevented by nicotine. Nicotine-mediated protection involved MEK/Erk1/2 activity, as pharmacological inhibition of MEK activity in nicotine-treated hippocampal slices caused synaptic activity to decrease similarly to that with NMDA treatment alone (Ferchmin et al., 2003). Erk1/2 activity has also been implicated in estrogen neuroprotection of cortical neurons after glutamate toxicity. Neuronal death was scored by assaying levels of lactose dehydrogenase (LDH) present in the media as loss of plasma membrane will cause a leakage of LDH from the cell. Five minute pretreatment with estrogen caused a 20% decrease in LDH release in primary cortical neurons, but this decrease was abolished with the application of a MEK inhibitor, PD98059 (Singer et al., 1999). Interestingly, inhibition of Erk1/2 activity in axons of intact sympathetic neurons supplied with NGF has no effect on their survival (MacInnis and Campenot, 2005). These results suggest that Erk1/2 activity is necessary

for survival only in injured neurons (reviewed by (Hetman et al., 2002)), and raises the possibility that Erk1/2 activity may be involved in maintaining axonal survival as well.

1.7. Compartmented culture system and sympathetic neurons:

Cell bodies and axon terminals of neurons often exist and function in different fluid environments in an intact animal. The compartmented culture system provides the opportunity to recreate this scenario *in vitro* (MacInnis and Campenot, 2005). Three compartments are created using a Teflon divider that is sealed to a tissue culture dish by silicon grease. This seal results in the ability to create three separate and distinct chemical environments that will not mix with adjacent compartments. This chemical separation lasts for weeks as the silicon forms an effective seal between the Teflon and the bottom of the tissue culture dish. Cell bodies are plated in center compartments and grow axons along collagen tracks that extend into adjacent compartments. This growth into distal compartments creates compartments that contain axons but no cell bodies, while center compartments contain both cell bodies and proximal axons (Figure 1.3).

Superior cervical ganglion neurons, which are used in these studies, are sympathetic neurons that are part of the PNS and have been extensively studied as they are very amenable to experimentation and grow quickly in culture. Sympathetic neuron survival is regulated by NGF that is supplied by the targets they innervate. This regulation by NGF controls the development and maintenance of neuronal connections and occurs by retrograde signaling, where a signal travels from the axon terminal toward the cell body (Hendry et al., 1974). Several types of neurons, including sympathetic neurons, can have processes that extend up to 1 meter or more away from the cell bodies.

This physical separation of axon and cell body may result in different environments favouring different mechanisms of survival, and understanding the mechanisms behind axon degeneration will have important implications for neurodegenerative diseases, peripheral neuropathies, and neuronal trauma.

Degeneration of cortical neuron axons could also provide insights to a variety of neurodegenerative diseases and neuronal trauma, but growing cortical neurons in culture is difficult. Ivins et al. (Ivins et al., 1998) used a variety of substrates to grow hippocampal neurons in compartmented cultures so they could assess the toxic effects of local Aβ application on cortical axons. Even on the most effective growth substrate, poly-D-lysine/laminin-2, cortical axons only grew a short distance into the distal compartments (1000μm at most) under a very thin dividing barrier (approximately 120μm). Given this short growth distance, if cortical neurons were used in our compartmented cultures very little axon material, if any, would grow into the distal axon compartment due to the wider dividing barrier (1mm). This wider barrier would reduce the amount of axon material in the distal axon compartment making it very difficult to perform any experimentation. One example would be western blotting which requires a certain amount of material to perform, and using cortical neurons would require the use of a great many more cultures compared to sympathetic neurons. Sympathetic neurons grow much longer axon processes in culture that can easily cross the 1mm dividing barrier, and can grow millimeters into distal axon compartments. The larger amount of axonal material in distal compartments using sympathetic neurons would resolve the issue of amount of material needed to perform experimental assays, such as western blotting and quantification of the MTT assay.

Understanding the mechanisms behind axonal degeneration may give new insights into neuronal death resulting from injury and disease. The long-held belief that axons degenerated passively due to the loss of necessary transported materials from the neuronal soma has undergone a complete change in viewpoint with the discovery of the naturally occurring murine mutation, Wlds. Although the exact mechanisms that allow this mutation to cause delays in axon degeneration are still unknown, the current literature focuses on a chimeric fusion protein that contains a portion of an E4 ligase, Ube4b, as well as a functional Nmnat1. It was originally assumed and demonstrated experimentally that the delays in axon degeneration seen in Wlds-expressing neurons were the result of problems with the UPS due to the incomplete E4 ligase (Zhai et al., 2003). Focus has shifted to the role of Nmnat1 in axon degeneration and it has been suggested that only when functional Nmnat1 is overexpressed, either as a part of the chimeric Wlds protein or alone, are there delays in axon degeneration (Araki et al., 2004, Wang et al., 2005). Furthermore, Erk1/2 activity has been suggested to be necessary in survival of proteasome inhibited transected axons (MacInnis and Campenot, 2005). This raises the question of whether Erk1/2 activity is also involved in Wlds or NAD-mediated survival of transected axons.

Using compartmented cultures, mechanisms of Wallerian degeneration can be studied in distal axons without mechanically disturbing distal axons during cell body removal. Neurons are originally plated in the center compartment and grow axons into adjacent distal compartments. Cell body/proximal axon removal, a process referred to as transection, has no affect on distal axon adherence due to the physical separation of the cell bodies/proximal axons. Using these features the issue of whether NAD mediates its

protective effects through the cell bodies or axons can be experimentally studied in transected axons to determine where NAD mediates it protective effects.

Axons that have been transected from their neuronal cell body are protected from degeneration with pharmacological inhibition of the proteasome (Zhai et al., 2003, MacInnis and Campenot, 2005). Interestingly, exogenous NAD also protects transected axons from degeneration (Araki et al., 2004, Wang et al., 2005). This suggests that there are multiple ways of preventing transected axons from undergoing Wallerian degeneration, and the MEK/Erk signaling pathway may be necessary to maintain this protection in NAD-treated, transected axons as in proteasome inhibitor-treated, transected axons (MacInnis and Campenot, 2005).

1.8. Specific aims and hypothesis:

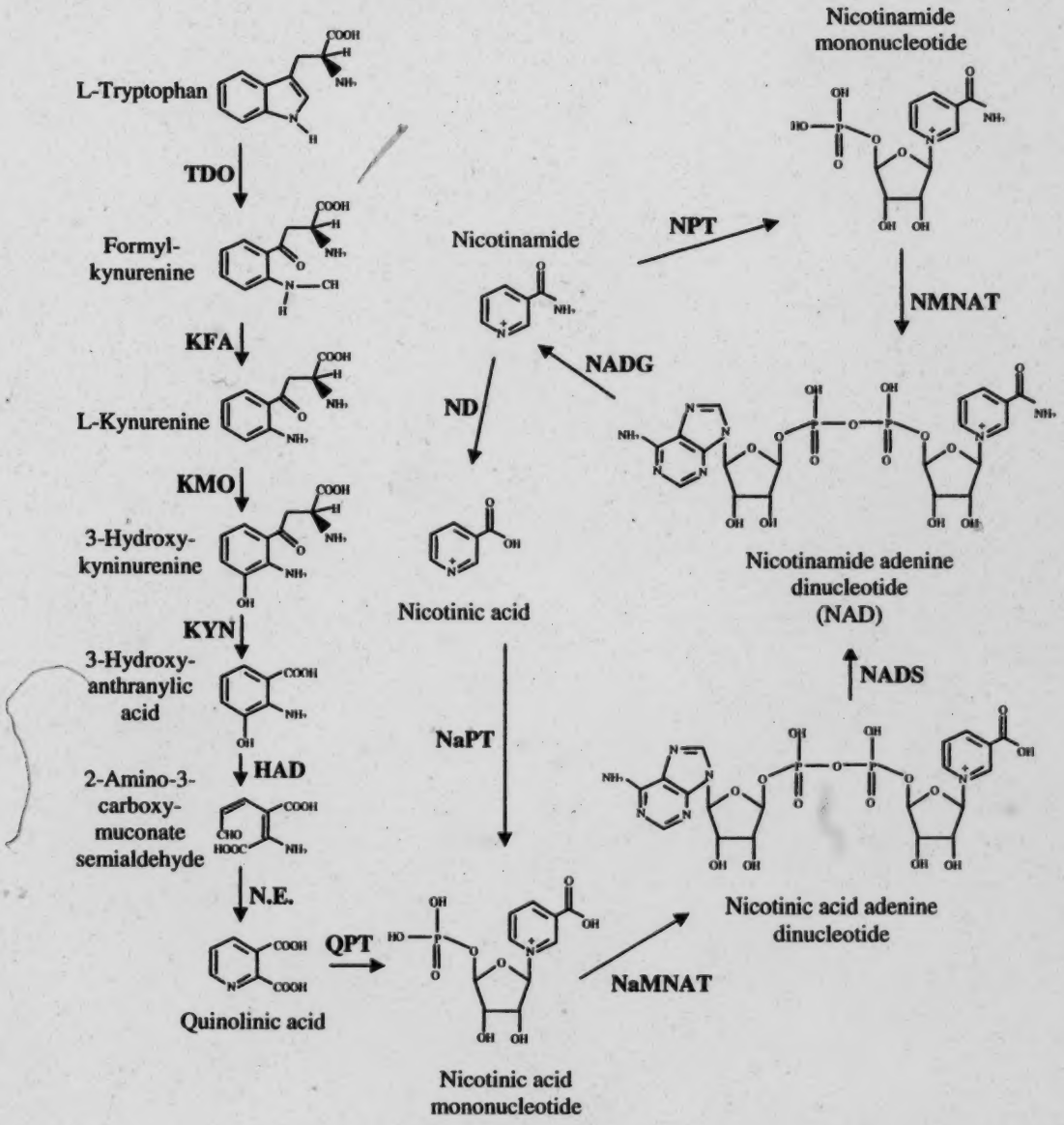
1. I set out to determine if NAD applied to sympathetic neurons could protect their distal axons from Wallerian degeneration. I hypothesized that treatment of the cell bodies/proximal axons before transaction would not result in protection of distal axons, and that treatment of the distal axons either before or after transaction could provide distal axon protection.

2. I hypothesized that severed distal axons that were protected from Wallerian degeneration would be dependent upon activation of the MEK/Erk pathway for their survival. To verify this I determined if phosphorylated Erk1/2 persists in the protected axons and if block of MAP kinase activity would cause the axons to degenerate.

3. I hypothesized that Erk1/2-mediated protection of transected axons functions through a downstream target, p90Rsk. I set out to determine if block of p90Rsk activity would cause protected, distal axons to degenerate.

4. I hypothesized that cytosolic sirtuins used exogenous NAD to mediate NADs protective effects. I set out to determine if block of sirtuin function would cause protected, distal axons to degenerate.

Figure 1.1 NAD *de novo* and salvage pathways in mammals. A diagrammatic representation of the *de novo* and salvage pathways in mammals. The *de novo* pathway uses L-Tryptophan as a starting substrate to form quinolinic acid. At this point the *de novo* pathway feeds into the salvage pathway with quinolinic acid being turned into nicotinic acid mononucleotide which is further modified into NAD, broken down and recycled.
Abbreviations used: TDO-tryptophan 2,3-dioxygenase; KFA-kynurenine formamidase; KMO-kynurenine 3-hydroxylase; KYN-kynureninase; HAD-3-hydroxyanthranilate 3,4-dioxygenase; N.E.-non-enzymatic; QPT-quinolinate phosphoribosyltransferase; NaPT-nicotinate phosphoribosyltransferase; NaNMAT-nicotinate mononucleotide adenyltransferase; NADS- NAD synthetase; NMNAT- nicotinamide mononucleotide adenyltransferase; NPT-nicotinamide phosphoribosyltransferase; ND- nicotinamide deamidase; NADG-NAD glycohydrolase



Adapted from Magni et al. (2004a) and Grubisha et al. (2005)

Figure 1.1. NAD *de novo* and salvage pathways in mammals

Figure 1.2. The Erk1/2 signaling cascade. Raf kinase causes the downstream phosphorylation and acitvation of MEK1/2. MEK1/2 in turn phosphorylates and activates Erk1/2. Once Erk1/2 becomes activated it can affect various processes in the cell through downstream targets that result in growth, differentiation and cell cycle regulation. Depicted is the downstream target p90Rsk, which inactivates BAD by maintaining its phosphorylation and preventing BADs pro-apoptotic effects. Also shown are the inhibitors U0126 and Ro-31-8220. U0126 prevents MEK1/2 phosphorylation by Raf and Ro-31-8220 prevents p90Rsk phosphorylation by Erk1/2.

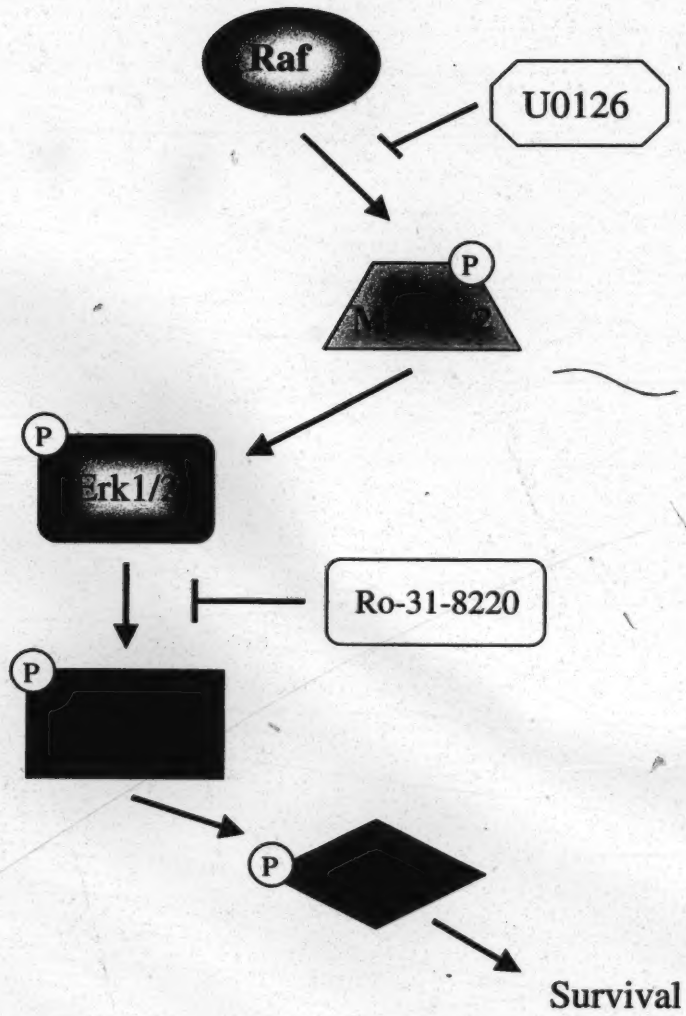
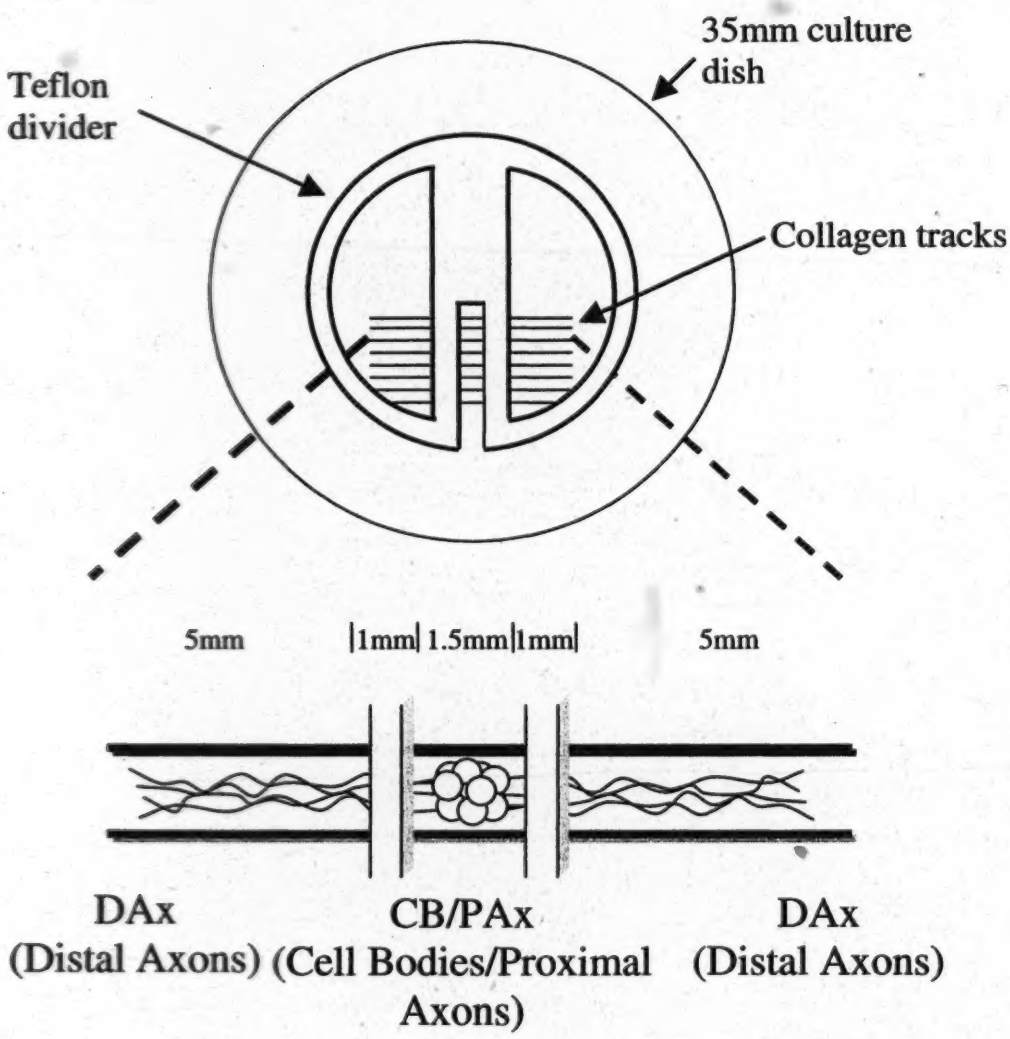


Figure 1.2. The Erk1/2 signaling cascade

Figure 1.3 Schematic of a 3 compartment neuron culture. Parallel scratches are made on a collagen-coated 35mm tissue culture dish. The dish is separated into 3 compartments by a Teflon divider that is sealed to the surface of the tissue culture dish by silicon grease. Sympathetic neurons are plated in the center compartment and extend axons along the tracks, beneath the divider wall and into the adjacent compartment. Below the compartment culture is an enlargement of a single track.



Adapted from R.B. Campenot and S.A. Mok

Figure 1.3. Schematic of a 3 compartment neuron culture

2. Experimental Methods

2.1. Culture Methods:

2.1.1. Compartment Culture Construction:

35mm plastic culture dishes (Falcon, Franklin Lakes, NJ) were coated with a 20% collagen solution prepared in double distilled water (collagen was prepared from adult rat tails provided by University of Alberta Health Sciences Lab Animal Services). After the collagen dried, the coated dishes were scratched with a pin rake to create 500μm lanes in the collagen coating. Under a Zeiss stereoscope (Zeiss, Toronto, ON), a droplet of $L15CO_2$ base medium (500ml L-$15CO_2$ base medium (L-$15CO_2$; 10.8mg/ml [Gibco Laboratories, Grand Island, NY], 0.04mg/ml imidazole [Sigma-Aldrich, St. Louis, MO], $7.8x10^{-3}$mg/ml phenol red [Sigma-Aldrich, St. Louis, MO], 3ml stable vitamin mix {stable vitamin mix; 3mg/ml L-aspartic acid, 3mg/ml L-glutamic acid, 3mg/ml L-proline, 3mg/ml L-cystine, 1mg/ml p-aminobenzoic acid, 1mg/ml β-alanine, 0.4mg/ml vitamin B12, 2mg/ml myo-inositol, 2mg/ml choline chloride, 5mg/ml fumaric acid, 0.08mg/ml coenzyme A, 0.04mg/ml d-biotin, 1mg/ml DI6,8 thioctic acid [all products from Sigma-Aldrich, St. Louis, MO], dissolved in double distilled H_2O} in double distilled H_2O, pH 7.35,), 20g methyl cellulose [Sel-Win chemicals, Vancouver, BC], 10ml of D(+)glucose [300mg/ml, Sigma-Aldrich, St. Louis, MO], 5ml glutanime [0.02mg/ml, Sigma-Aldrich, MO], 5ml penicillin-streptomycin [10mg/ml, Sigma-Aldrich, St. Louis, MO], 5ml fresh vitamin mix {0.5mg/ml 6,7-dimethyl-5,6,7,8-tetrahydropterine, 0.25mg/ml glutathione} [both from Sigma, Aldrich, St. Louis, MO]) containing 50ng/ml 2.5S NGF [Alamone Labs, Jerusalem, Israel] was added to the scratches. After this, sterile Teflon dividers (Tyler Research Instruments, Edmonton, AB) were coated with silicon grease and applied

to the dishes so that the tracks and medium ran through all compartments of the dividers. This allowed for neurons plated in the center compartment to extend axons into the distal compartments, but still provide a seal between each compartment, allowing for separate fluid environments (Figure 1.3).

2.1.2. Dissociation of ganglia:

Superior cervical ganglion neurons were dissected from 0-2 day old Sprague–Dawley rats supplied by the University of Alberta Health Sciences Lab Animal Services. After removal, ganglia were incubated in collagenase (1mg/ml [Sigma-Aldrich, St. Louis, MO] in PBS {phosphate buffer saline;8mg/ml NaCl, 0.2mg/ml KCl, 0.24mg/ml KH_2PO_4, 1.44mg/ml NaH_2PO_4 in double distilled H_2O, pH 7.2}) for 25 minutes at 37 degrees Celsius to break apart collagen holding the ganglia together. This reaction was halted with the application of trypsin (10mg/ml [Sigma-Aldrich, St. Louis, MO]) for 5 minutes at 37 degrees Celsius. Ganglia were then rinsed in sterile PBS which was warmed to 37 degrees Celsius and resuspended in 8ml L-15CO_2 medium supplied with 800μL 2.5% rat serum (prepared from the blood of adult male rats and diluted in double distilled H_2O). Ganglia were centrifuged for 1 minute at 12 times gravity and the supernatant aspirated. The ganglia were resuspended in L15CO_2 medium and were mechanically separated by pipetting. Once the ganglia had been dissociated, the ganglia and media was filter sterilized using a sterile 0.5μm filter. This filtered media was centrifuged at 23 times gravity for 3 minutes to pellet down the neurons.

2.1.3. Culture Medium:

After neurons were dissociated, they were resuspended in L15CO_2 base medium that contained 10 ng/ml 2.5S NGF (Alamone Labs, Jerusalem, Israel) and 2.5% rat serum. This medium also contained 1 ml cytosine arabinoside (0.25mg/ml [Sigma, St. Louis, MO]) and 1ml vitamin C (5mg/ml, pH 5-6, [Sigma-Aldrich, St. Louis, MO]). The cytosine arabinoside eliminated non-neuronal cells that were present in the culture during the first 7 days. This neuron-containing media was plated in the center compartment and the distal compartments were supplied with L-15CO_2 base medium containing no serum and 50 ng/ml 2.5S NGF. Three-compartmented cultures were maintained for 10–11 days, with solution changes after 7 days, where distal compartments received fresh L-15CO_2 base media that contained 50ng/ml 2.5S NGF, while the center compartment was given L-15CO_2 base medium that contained 2.5ml of 2.5% rat serum and 1ml vitamin C. Treatment conditions are indicated in the Results section, and treatment groups within each experiment consisted of cultures from the same plating of neurons and were maintained under identical conditions until the time of the experiment.

2.2. Reagents and Inhibitors:

Nicotinamide adenine dinucleotide (NAD) (Sigma-Adlrich, St. Louis, MO) was prepared as a stock solution of 1mM in double distilled water. Dimethylsulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of inhibitors were prepared as follows: U0126 (50 mM in DMSO; Promega Corporation, Madison, WI), Sirtinol (20mM in DMSO; Calbiochem, San Diego, CA) and Ro-31-8220 (5mM in DMSO; Calbiochem, San Diego, Ca). Dilutions were made directly into

working culture medium. Control groups were given an equivalent volume of vehicle, which never exceeded a volume of 0.06% of the total treatment volume, which had no detrimental effects on the neurons. In experiments using multiple concentrations of Ro-31-8220, a volume of DMSO equivalent to the highest concentration of drug was used in vehicle only treatments.

2.3. Axon Metabolic Assay:

After experimental treatments distal axons were incubated for 45 min at 37 degrees Celsius with CellTiter 96 One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI), a derivative of the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in which cell survival is measured by the conversion of MTT from a yellow liquid to a blue formazan crystal by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983). For quantitative analysis of formazan development, axons were harvested from distal compartments using 50μL ice-cold PBS by scratching and washing axons off of the dish substratum. Axonal material was then collected and pelleted at 13,100 times gravity. Supernatant was removed and the formazan-containing axon pellets were dissolved in 50μL of Solubilizing/Stop solution. The extent of color development was measured quantitatively by 96 well plate-reading at a wavelength of 570 nm. Images of stained axons were obtained first by halting the assay reaction by applying ice-cold PBS that contained 0.04% methylcellulose (which reduced sheering forces) to distal axon compartments then incubating for 1h at 4°C. Images were then taken using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Nikon Digital Camera DX-1200 (Nikon Canada, Toronto, ON).

2.4. Immunoblotting:

Following experimental treatment, cultures to be analyzed by immunoblotting were rinsed with ice-cold TBS (Tris buffer saline; 2.42mg/ml Tris [Sigma, St. Louis, MO], 8mg/ml NaCl [Sigma-Aldrich, St. Louis, MO], pH 7.5) containing 18.5 mg/mL sodium orthovanadate (Sigma-Aldrich, Oakville, ON) and 40 mg/mL sodium fluoride (Sigma-Aldrich, Oakville, ON). Distal axons were then harvested directly into 3 X SDS (sodium dodecyl sulphate) sample buffer by scratching the collagen substratum with a micro gel loading tip. Each group consisted of 4-6 compartments worth of axon material. β-mercaptoethanol (BioShop, Burlington, ON) was added to the sample solution equivalent to 5% of the total sample volume. Sample solutions were boiled for 5 min at 100 degrees Celsius and separated by electrophoresis on 12% SDS-PAGE (SDS-polyacrylamide gel electrophoresis) gels. Gels were run at 0.65 amps through the stacking gel and 0.75 amps through the running gel. Proteins were transferred to Immobilon-P PVDF membrane (Millipore Corporation, Bedford, MA) at 30 volts overnight.

PVDF membranes were immunoblotted using the following primary antibodies at the indicated dilutions: rabbit monoclonal anti-phospho-Erk1/2 (Thr202/Tyr204) antibody (Cell Signaling Technology, Beverly, MA) was used at a 1:1000 dilution, polyclonal anti-Erk1 antibody (detects Erk1 and Erk2) (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:10,000 diution, polyclonal anti-phospho-p90 ribosomal S6 kinase antibody (Cell Signaling, Beverly, MA) was used at a dilution of 1:1000, polyclonal anti-Rsk1/2/3 (Cell Signaling, Beverly, MA) was used at a dilution of 1:1000, mouse monoclonal anti-β-actin (Sigma-Aldrich, St. Louis, MO) was used at a 1:1000

dilution and mouse monoclonal anti-β-tubulin (Sigma-Aldrich, St Louis, MO) was used at a 1:1000 dilution.

Membranes were first blocked in 5% milk in TBS-T (TBS with 0.1% Tween 20[Fischer Scientific, pH 8.0]) for 1 hour at room temperature. The membrane was then washed for 5 minutes in TBS-T 3 times. Following these washes, primary antibodies were diluted in a blocking solution containing 5% BSA (bovine serum albumin [Sigma-Aldrich, St. Louis, MO]) in TBS-T and the membrane was incubated at room temperature for 1 hour or overnight at 4 degrees Celsius. After primary antibody incubation, membranes were rinsed for 5 minutes in TBS-T 3 times. Following these washes, the appropriate secondary antibodies (polyclonal goat IgG anti-mouse IgG conjugated to horseradish peroxidase [Pierce, Rockford, IL] at a 1:2000 dilution, or polyclonal goat IgG anti-rabbit IgG conjugated to horseradish peroxidase [Pierce. Rockford, IL] at a 1:2000 dilution in blocking solution containing 5% milk) were added to membranes for 1 hour at room temperature. After secondary antibody incubation, membranes were again rinsed 3 times for 5 minutes each in TBS-T. Membranes were then treated with 1ml enhanced chemiluminescence SuperSignal West Dura Substrate or SuperSignal West Femto (Pierce, Rockford, IL) for 3 minutes and exposed with Kodak BioMax MR film (Kodak, Rochester, NY).

3. Results

3.1. Treatment of distal axons with NAD protects transected axons from Wallerian degeneration:

Due to the conflicting evidence presented by Araki et al. (Araki et al., 2004) and Wang et al. (Wang et al., 2005) of NAD protecting transected axons from Wallerian degeneration through either the cell bodies or axons, compartmented cultures were used to explore NAD-mediated protection. As stated above, compartmented cultures allow separate chemical treatments of cell bodies/proximal axons and distal axons. Taking advantage of the separate chemical environments, NAD was applied to cell bodies/proximal axons or distal axons of 10–11-day-old neurons maintained in compartmented cultures to determine whether NAD protection of transected axons from Wallerian degeneration acted through the cell bodies or the axons. For cell body/proximal axon treatment, cell bodies/proximal axons were pretreated for 24 hours with various concentrations of NAD (1μM-20mM) before being removed. Treatment of distal axons included 24 hour treatment of distal axons before cell body/proximal axon removal (pretreatment), 24 hour treatment of distal axons after cell body/proximal axon removal (post-treatment), or both NAD pre- and post-treatment. In all distal axon treatments, axons were either given medium containing NGF (50ng/ml) or NGF plus NAD (1, 5, 10 or 20mM). Wallerian degeneration of distal axons was observed 24 hours after cell bodies/proximal axons were removed.

As axons grow into distal compartments, individual axons coalesce into cables on the collagen tracks. These cables are also surrounded by other axons that adhere in the periphery of the collagen track. As axons degenerate, peripheral axons detach and

collapse around the main cable and cables begin to retract until the axon eventually deteriorates. Using these morphological signs of axon degeneration and the MTT assay to assess mitochondrial function (in which MTT tetrazolium dye is reduced to a dark blue formazan precipitate by active mitochondrial succinate dehydrogenase), images of transected axons were examined for Wallerian degeneration after treatment of cell bodies/proximal axons and distal axons.

Wallerian degeneration of distal axons was delayed by application of 5-20mM NAD to the distal axons, whether NAD was applied before or after cell body/proximal axon removal (Figure 3.1A). Peripheral axons had detached and collapsed around the main cable, but the main cable was still well adhered to the collagen substratum. These axons also displayed reduced MTT reactivity (Figure 3.1). NAD application to distal axons delayed Wallerian degeneration in transected distal axons and MTT reactivity was increased 8-15% compared to distal axons of untreated, transected controls (Figure 3.1B). These results suggest exogenous NAD application to distal axons, before or after transection, delayed Wallerian degeneration, implicating a local effect on the axons and not an effect that was transmitted from the cell bodies.

NAD post-treatment and pre-/post-treatment with 1mM NAD also protected transected distal axons, but pretreatment of axons with 1mM NAD alone did not. This suggests that this concentration of NAD may not be protective by pretreatment alone or the effective concentration of NAD may not last the duration of the experiment. Regardless of the differences between the timing of the treatments, NAD mediates its protective effects when locally applied to transected axons.

Distal axons of neurons whose cell bodies/proximal axons were pretreated with NAD were not protected from Wallerian degeneration following transection (Figure 3.2). Both transected axons of pretreated and untreated cell bodies/proximal axons had disintegrated and were poorly attached to the substratum, if at all (Figure 3.2A). NAD pretreatment of cell bodies and proximal axons did not delay Wallerian degeneration of transected distal axons implying the protective effects of exogenous NAD application are not mediated through the cell bodies, as previously reported (Araki et al., 2004).

3.2. NAD mediated protection of transected axons requires MEK/Erk kinase activity:

The MEK/Erk signal transduction pathway is regulated by protein phosphorylation. Phosphorylation results in protein activation and further phosphorylation of down-stream targets. MEK/Erk activity is involved in locally regulating axon growth and morphology (Atwal et al., 2000) and is involved in survival of proteasome inhibited, transected axons (MacInnis and Campenot, 2005). This raised the question as to whether inhibition of Erk1/2 activity would prevent the protective effects of exogenous NAD applied to transected distal axons. Transected axons given 50ng/ml NGF were treated for 24 hours with NAD alone (1 or 5mM), NAD and 50μM U0126 (a MEK inhibitor), or left untreated. Inhibition of MEK activity resulted in a loss of mitochondrial reactivity in distal axons treated with 1 and 5mM NAD (Figure 3.3). These axons degenerated similarly to untreated, transected controls, displaying morphological signs of degeneration (Figure 3.3A). Transected distal axons given U0126 alone also degenerated similarly to distal axons of transected controls. Again, transected distal axons given NAD alone displayed increased mitochondrial reactivity with 1mM

and 5mM NAD producing 14 and 21% MTT reactivity compared to distal axons of untreated, transected controls (Figure 3.3B). The loss of NAD-mediated protection that occurs with NAD and U0126 treatment suggests that MEK activity plays a role in axonal survival and its inhibition causes NAD-protected, transected axons to degenerate normally. These results combined with the previous report indicating the importance of MEK/Erk activity in proteasome inhibited axon protection suggests that the MEK/Erk pathway may be a general signaling pathway involved in maintaining axonal function following transection.

3.3. NAD treatment of transected distal axons maintains Erk1/2 phosphorylation up to 24 hours:

The role of the MEK/Erk pathway in NAD-mediated axon protection was further explored by determining how inhibition of MEK with U0126 affected MEK kinase activity. MEK inhibition was assessed by assaying the phosphorylation states of Erk1/2, downstream targets of MEK1/2 kinases. Immunoblot analysis was performed using distal axon lysates of intact, untreated axons and transected axons treated with NAD alone (1 or 5mM), NAD and 50μM U0126, or left untreated for 4, 8, or 24 hours. All distal axon treatments were also given 50ng/ml NGF throughout incubations. After transection Erk1/2 phosphorylation was present in all groups except those given the MEK inhibitor (Figure 3,4A), but was maintained only in NAD-treated, transected axons at 8 hours (Figure 3.4B, lane 4 and 6). Erk1/2 phosphorylation was still present in NAD-treated, transected axons at 24 hours (Figure 3.4C, lane 4 and 5). Interestingly, Erk1/2 phosphorylation displayed a concentration dependent increase as 5mM NAD treated

distal axons had a stronger Erk1/2 phosphorylation band compared to 1mM NAD treated distal axons (Figure 3.4C, lane 4 vs. 5). However, Erk1/2 phosphorylation was lost by 8 hours in untreated, transected axons (Figure 3.4B, lane 2) and was absent or greatly reduced in transected axons treated with U0126 (with or without NAD) at all time points (Figure 3.4). Levels of Erk1/2 protein were unchanged at 8 hours, indicating the decrease in phosphorylation in untreated, transected axons was due to a loss of protein phosphorylation and not the Erk1/2 proteins. These results demonstrate that NAD treatment is able to maintain Erk1/2 phosphorylation in transected axons, similar to what is seen with proteasome inhibitor treatment of transected axons (MacInnis and Campenot, 2005). This suggests that regardless of how axon degeneration is delayed in transected axons, the Erk1/2 signal transduction pathway is necessary for transected axon survival.

3.4. Ro-31-8220 increases mitochondrial activity of NAD-treated, transected axons and increases Erk1/2 phosphorylation:

To determine potential downstream targets of Erk1/2 activity which may play a role in axonal survival, another protein kinase inhibitor, Ro-31-8220, was used. Ro-31-8220 has been shown to inhibit phosphorylation of p90 ribosomal S6 kinase (p90Rsk) (Alessi, 1997), a downstream target of Erk1/2, which inactivates via phosphorylation various pro-apoptotic proteins, such as BAD (Nguyen et al., 2005). When BAD becomes dephosphorylated, it activates and translocates to the mitochondria resulting in mitochondrial destabilization. Transected distal axons given 50ng/ml NGF were treated for 24 hours with Ro-31-8220 alone (1 and 5μM), NAD alone (5mM), Ro-31-8220 and NAD, left untreated, or left untreated and intact. As seen previously, transected axons

treated with NAD had increased MTT reactivity compared to distal axons of untreated, transected controls (Figure 3.5A). Surprisingly, treatment of transected axons with Ro-31-8220 and NAD doubled MTT reactivity at both Ro-31-8220 concentrations (1 and 5μM, respectively) compared to NAD treatment alone (Figure 3.5B). Transected distal axons given Ro-31-8220 alone degenerated similarly to distal axons of transected controls.

The above results were unexpected as inhibition of p90Rsk should have caused the activation of downstream proteins involved in destabilizing the mitochondria, such as BAD, and cause normal degeneration of the axon. Therefore, immunoblot analysis was performed at 24 hours after drug treatment to assess the effectiveness of Ro-31-8220 at inhibiting p90Rsk phosphorylation. Following the same experimental treatments as above, it was demonstrated that a small amount of phosphorylated p90Rsk was present in NAD- and Ro-31-820-treated, transected axons (Figure 3.5C, lanes 6 and 7) suggesting p90Rsk was not completely inhibited. Interestingly, p90Rsk phosphorylation is increased in Ro-31-8220 and NAD-treated, transected axons compared to NAD treatment alone of transected axons (Figure 3.5C, lanes 6 and 7 vs. 5). Similar increases were also seen in Erk1/2 phosphorylation in transected axons treated with NAD and Ro-31-8220 compared to NAD alone (Figure 3.5C, lanes 6 and 7 vs. 5). Loss of Erk1/2 phosphorylation and p90Rsk phosphorylation of Ro-31-8220 treated, transected axons and untreated, transected axons most likely reflects decreases in total protein levels and is confirmed by the β-actin loading control. Ro-31-8220 appears not to inhibit the phosphorylation of p90Rsk, a downstream target of Erk1/2, and also appears to have protective effects of its own, potentially mediated through inhibition of PKC or other kinases.

3.5. Sirtinol treatment increases mitochondrial activity of NAD-treated, transected axons, but axon survival is still dependent on MEK kinase activity:

Araki *et al.* (Araki *et al.*, 2004) demonstrated that NAD-mediated protection required SIRT1 function in transected axons of dorsal root ganglia neurons. SIRT1 is a protein deacetylase located in the nucleus that has been implicated in transcriptional silencing (Yeung 2004; Brunet et al., 2004; Motta et al., 2004; van der Horst et al., 2004; Yang et al., 2005) as well as deactivation of p53, a protein with pro-apoptotic function (Lou et al., 2001; Langley et al., 2002). It is possible that other SIRT family members with deacetylase function present in the cytosol may be involved in NAD-mediated protection of transected axons by acting on the similar targets present in the cytosol. If other cytosolic SIRT family members are involved in NAD-mediated protection, SIRT inhibition should lessen the protective effects of NAD. A preliminary result of SIRT inhibition by sirtinol (a general SIRT inhibitor) combined with NAD treatment caused an increase in MTT reactivity over NAD treatment alone. This result suggested SIRT inhibition improved mitochondrial reactivity of NAD-treated, transected axons, instead of reducing it. Due to this result, the effects of MEK kinase inhibition were also explored. To do this, axons given 50ng/ml NGF were transected and treated for 24 hours with NAD alone (1 and 5mM), NAD and 100μM sirtinol, 50μM U0126 in addition to NAD and sirtinol, sirtinol and U0126, sirtinol alone, left untreated or left untreated and intact. As seen before, NAD treated axons had a substantially increased MTT reactivity compared to distal axons of transected controls (Figure 3.6). Axons treated with NAD and sirtinol combined resulted in an increase of MTT reactivity over NAD treatment alone (Figure 3.6A). Addition of sirtinol with 1mM NAD doubled MTT reactivity compared to

treatment with 1mM NAD alone in transected axons (Figure 3.6B). Sirtinol tripled MTT reactivity in the presence of 5mM NAD in transected axons compared to treatment with 5mM NAD alone (Figure 3.6B). MEK inhibition using U0126 combined with NAD and sirtinol treatment of axons resulted in no detectable mitochondrial reactivity by 24 hours as did transected axons given only sirtinol, sirtinol and U0126, or left untreated (Figure 3.6A). Although sirtinol treatment was unable on its own to maintain function in transected distal axons, it did improve MTT reactivity of NAD treated axons (Figure 3.6A). However, this increase in reactivity was still reduced compared to axons of intact neurons. As was seen above, transected distal axons maintained by NAD or NAD and sirtinol were still dependent on MEK activity. These results again suggest that regardless of how transected distal axon function is preserved, MEK activity is required to maintain axon function.

3.6. Sirtinol and NAD treatment of transected distal axons maintains Erk1/2 phosphorylation up to 24 hours:

Next, MEK kinase activity was assayed in the presence of these inhibitors by assessing Erk1/2 phosphorylation by immunoblot analysis. Distal axon lysates were harvested from intact, untreated axons and transected axons treated with NAD alone (1 or 5mM), NAD /100μM sirtinol, NAD/sirtinol/50μM U0126, sirtinol/U0126, sirtinol alone or left untreated for 4, 8, or 24 hours. All distal axon treatments were also given 50ng/ml NGF throughout the incubations. Again, Erk1/2 phosphorylation was maintained at 8 hours (Figure 3.7B, lanes 5 and 8) and was still present at 24 hours after transection of NAD-treated axons (Figure 3.7C, lanes 5 and 8). Erk1/2 phosphorylation was also

maintained for 24 hours in NAD and sirtinol treated transected axons, but was increased compared to NAD treatment alone at 24 hours (Figure 3.7C, lane 5 vs. 6 and lane 8 vs. 9). Interestingly, Erk1/2 phosphorylation was lost by 8 hours in untreated, transected axons but not in transected axons treated with sirtinol alone (Figure 3.7B, lane 2 vs. 3). This maintenance of Erk1/2 phosphorylation at 8 hours with sirtinol treatment alone suggests that sirtinol may have protective effects which do not last the full 24 hour treatment (Figure 3.7C, lane 3). Again, levels of Erk1/2 protein were unchanged at 8 hours, indicating the decrease in phosphorylation in untreated, transected axons and sirtinol and U0126 treated, transected axons was due to a loss of protein phosphorylation and not the Erk1/2 proteins. These results again demonstrate that NAD is able to maintain Erk1/2 phosphorylation in transected axons, similar to Erk1/2 phosphorylation with proteasome inhibitors, and that phosphorylation is increased in the presence of sirtinol. These increases in phosphorylation correlate with increases in MTT reactivity.

Figure 3.1. Treatment of axons with NAD delays Wallerian degeneration. Distal axons given 50ng/ml NGF were either pretreated for 24 hours with the indicated concentrations of NAD before cell body/ proximal axon removal (pretreatment), treated with NAD after cell body/proximal axon removal (post-treatment), or both (pre- and post-treatment). Control culture distal axons were also given 50ng/ml NGF and left untreated before and after cell body/proximal axon removal or left intact and untreated.

A. – Bright field images of axons stained with MTT 24 hours after cell bodies and proximal axons have been removed. Cables of metabolically active axons remained attached to the substratum (arrow heads). Metabolically inactive axons cables tend to detach from the substratum (arrows). Results are representative of three experiments. The scale bar is 200μm.

B.– Quantification of MTT stained axons 24 hours after cell body and proximal axon removal. N=9, Data are compiled from 3 experiments each with 3 cultures per group. Values are presented as the mean absorbance ± SEM. Statistically significant differences between transected controls and treatment groups are indicated by three asterisks (Two-Sample Assuming Unequal Variances, $p<0.001$)

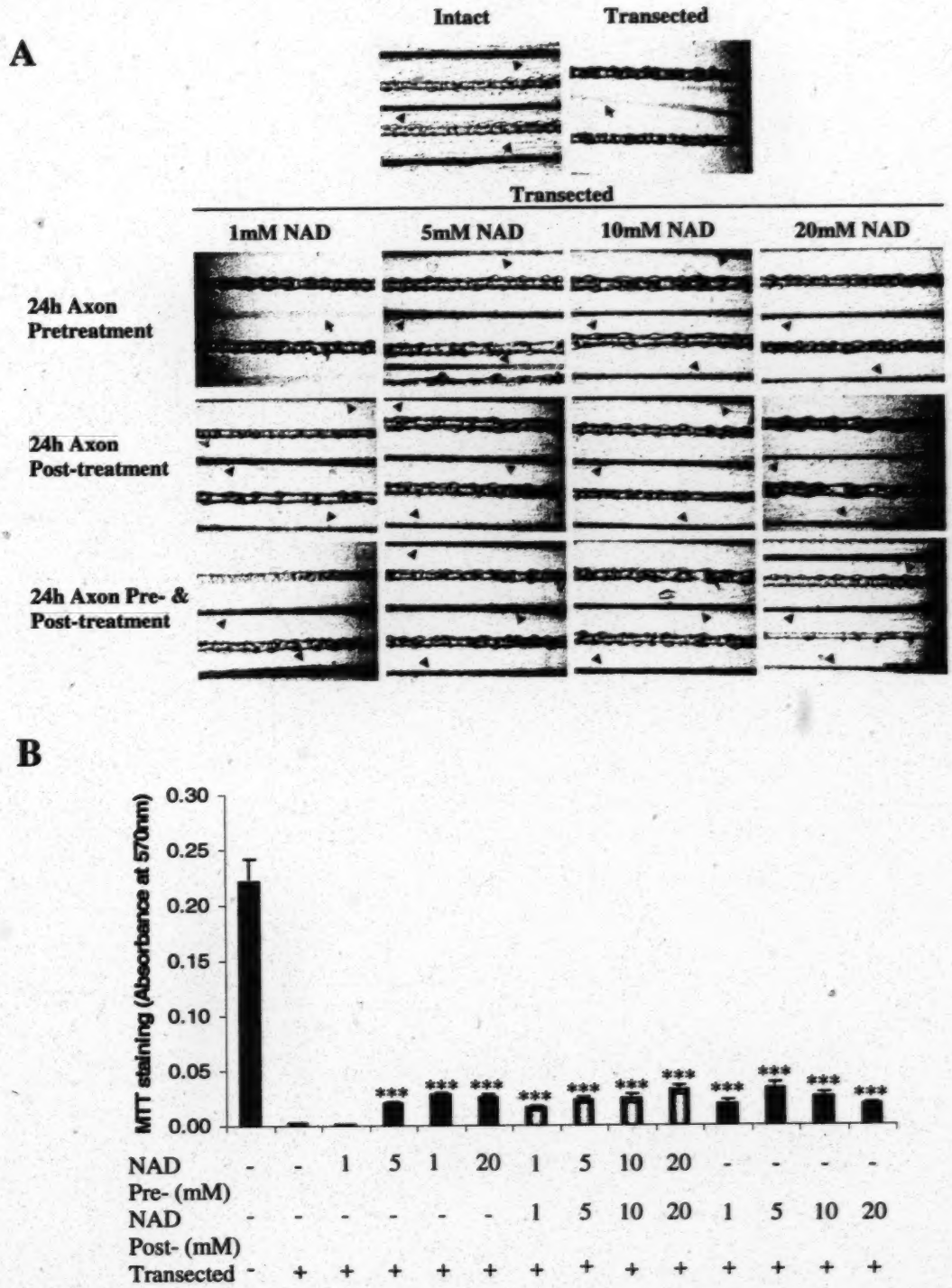


Figure 3.1. 24 hour Cell Body/Proximal Axon pretreatment with NAD does not prevent Wallerian degeneration

Figure 3.2. 24h Cell Body/Proximal Axon pretreatment with NAD does not prevent Wallerian degeneration. Neuronal cell bodies and proximal axons in the central compartment are treated for 24 hours with a NAD-containing media or untreated. After the pretreatment, the cell bodies and proximal axons are removed by transection and distal axons were given fresh media containing 50ng/mL NGF. Control culture cell bodies/proximal axons were left untreated before their removal or left intact and untreated.

A. – Bright field images of axons stained with MTT 24 hours after cell bodies and proximal axons have been removed. Cables of metabolically active axons remained attached to the substratum (arrow heads). Metabolically inactive axons cables tend to detach from the substratum (arrows). Results are representative of three experiments. The scale bar is 200μm.

B.– Quantification of MTT stained axons 24 hours after cell body and proximal axon removal. N=10-16, Data are compiled from 3 experiments each with 5-6 cultures per group. Values are presented as the mean absorbance ± SEM.

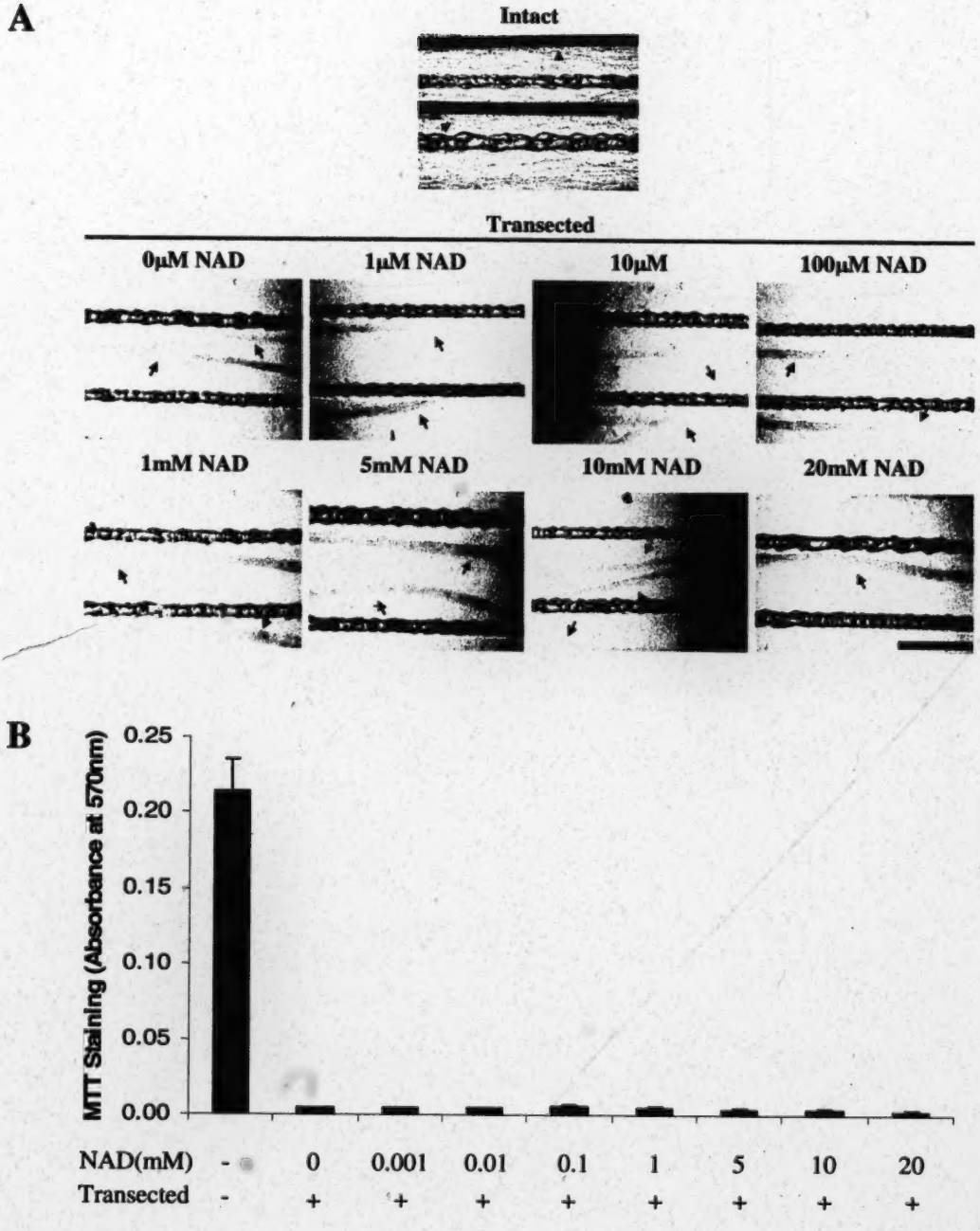


Figure 3.2. Treatment of Distal Axons with NAD delays Wallerian degeneration

Figure 3.3. MEK inhibition prevents NAD protection of transected axons. Distal axons given 50ng/ml NGF were treated with NAD alone, NAD and U0126, or U0126 alone. Control culture distal axons were also given 50ng/ml NGF and left untreated before and after cell body/proximal axon removal or left intact and untreated.

A.– Bright field images of axons stained with MTT 24 hours after cell bodies and proximal axons have been removed. The scale bar is 200μm.

B.– Quantification of MTT stained axons 24 hours after cell body and proximal axon removal. N=11, Data compiled from 3 experiments each with 3-4 cultures per group. Values are presented as the mean absorbance ± SEM. Statistically significant differences between transected controls and treatment groups are indicated by three asterisks (Two-Sample Assuming Unequal Variances, $p<0.001$)

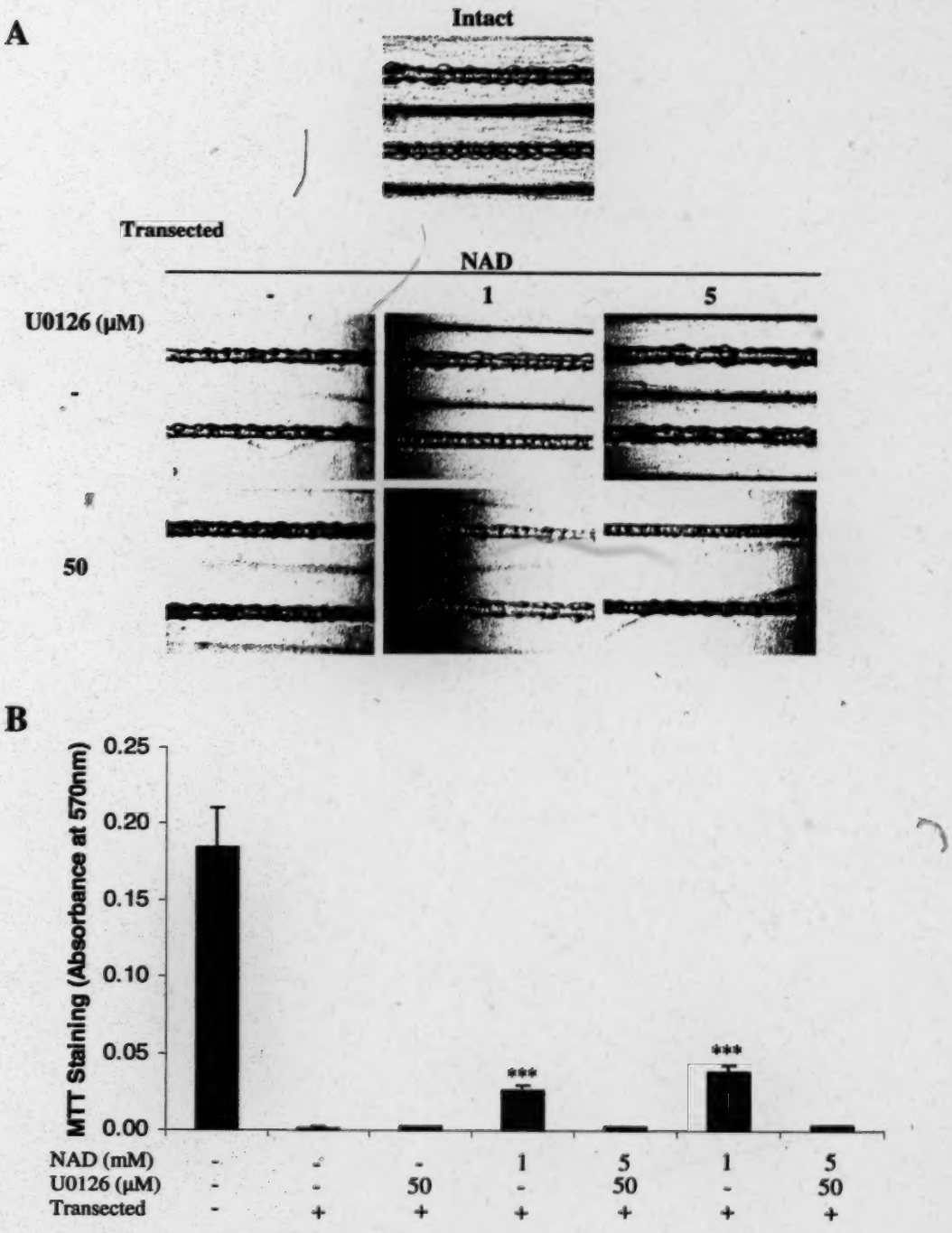


Figure 3.3. MEK inhibition prevents NAD-mediated protection of transected axons

Figure 3.4 NAD treatment of transected axons maintains Erk1/2 phosphorylation up to 24 hours. Distal axons given 50ng/ml NGF were treated with NAD alone, NAD and U0126, or U0126 alone. Control culture distal axons were also given 50ng/ml and left untreated before and after cell body/proximal axon removal or left intact and untreated. Axons we left treated for 4 hours (A), 8 hours (B), or 24 hours (C) before lysates were prepared for immunoblotting (4 distal compartments per lane). Phosphorylated Erk1/2 (upper panel), total Erk1/2(middle panel), and β-tubulin (lower panel) were visualized with anti-pErk1/2 mAB (1:1000), anti-Erk1/2 pAB (1:10,000) and anti-β-tubulin mAB (1:1000). Results are representative of 3 experiments.

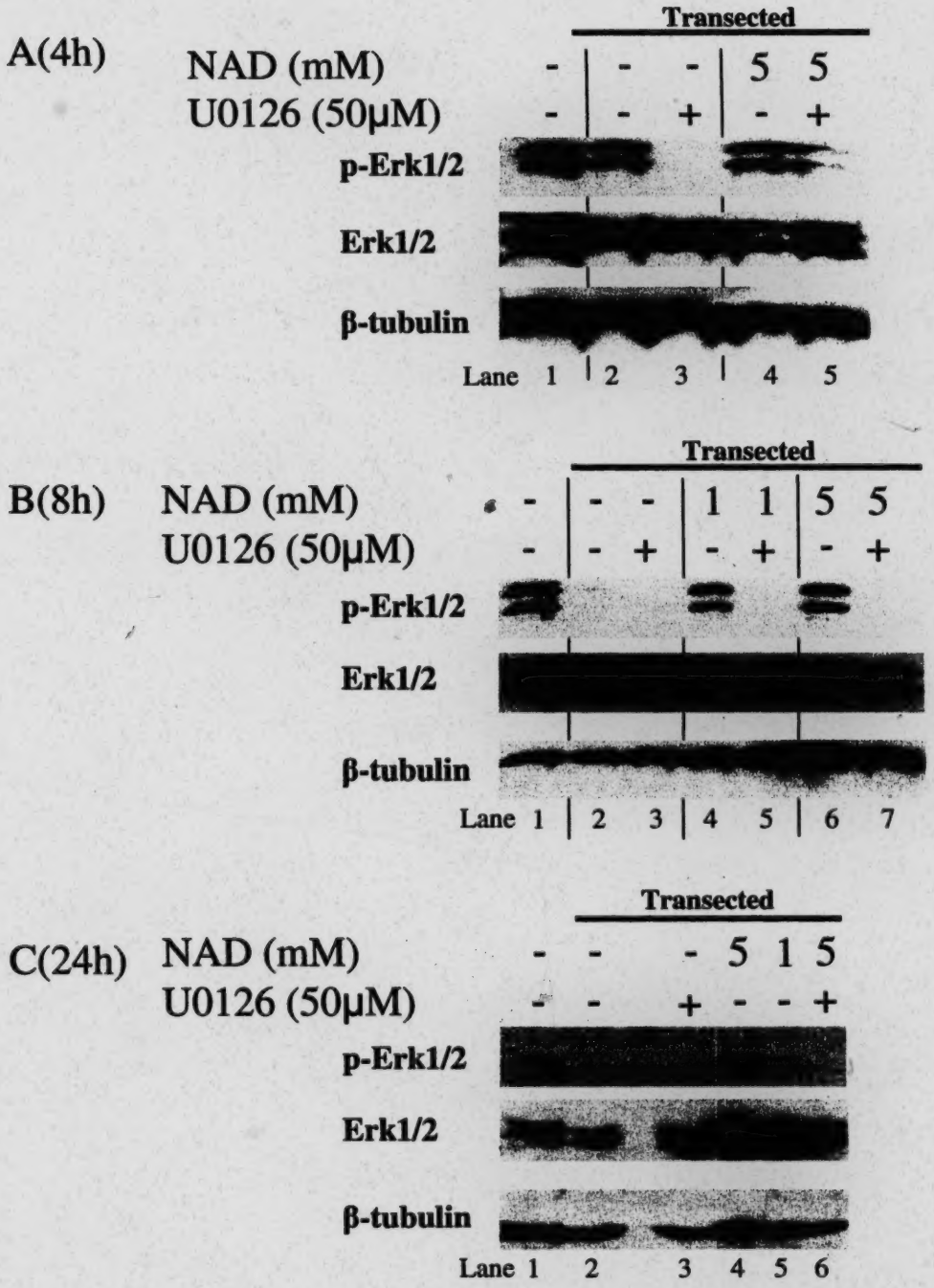


Figure 3.4. NAD treatment of transected axons maintains Erk1/2 phosphorylation up to 24 hours

Figure 3.5. Ro-31-8220 increases metabolic activity of NAD-treated, transected axons and increases Erk1/2 phosphorylation. Distal axons given 50ng/ml NGF were treated with NAD alone, Ro-31-8220 alone, or β-NAD and Ro-31-8220. Control culture distal axons were also given 50ng/ml and left untreated before and after cell body/proximal axon removal or left intact and untreated.

A.– Bright field images of axons stained with MTT 24 hours after cell bodies and proximal axons have been removed. The scale bar is 200μm.

B.– Quantification of MTT stained axons 24 hours after cell body and proximal axon removal. N=9, Data are compiled from 3 experiments each with 3 cultures per group. Values are presented as the mean absorbance ± SEM. Statistically significant differences between transected controls and treatment groups are indicated by three asterisks (Two-Sample Assuming Unequal Variances, $p<0.001$) and statistically significant differences between transected, NAD-treated and transected, NAD- and Ro-31-8220-treated groups are indicated by three pluses ($p<0.001$).

C.– Axons were treated for 24 hours before lysates were prepared for immunoblotting (4 distal compartments per lane). Phosphorylated Erk1/2 (top panel), total Erk1/2(top middle panel), phosphorylated p90RSK (middle panel), total RSK1/2/3 (bottom middle panel) and β-actin (bottom panel) were visualized with anti-pErk1/2 mAB (1:1000), anti-Erk1/2 pAB (1:10,000), anti-pp90RSK pAB (1:1000), anit-RSK1/2/3 pAB (1:1000) and anti-β-actin mAB (1:1000). Results are representative of 2 experiments.

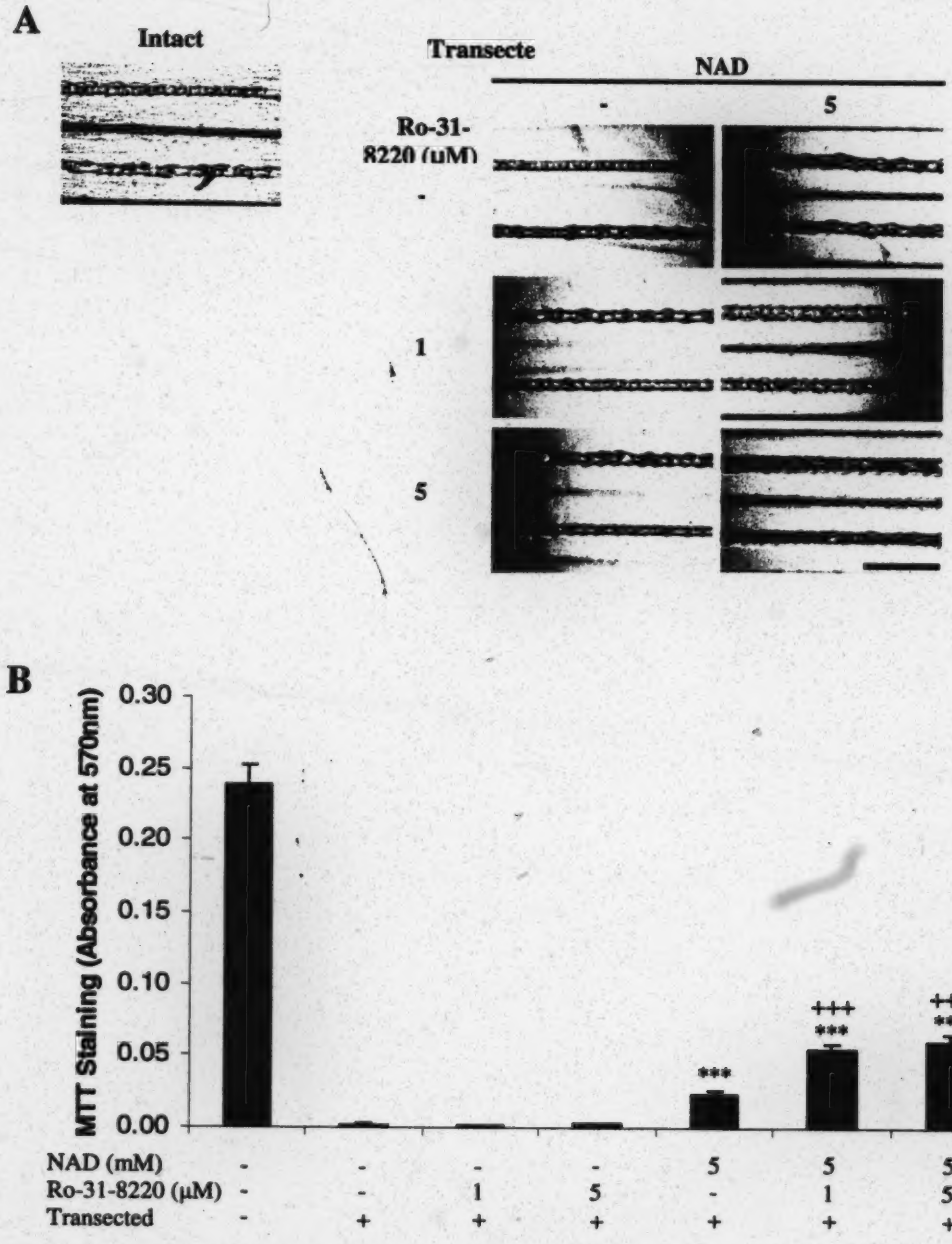


Figure 3.5. Ro-31-8220 increases metabolic activity of NAD-treated, transected axons and increases Erk1/2 phosphorylation

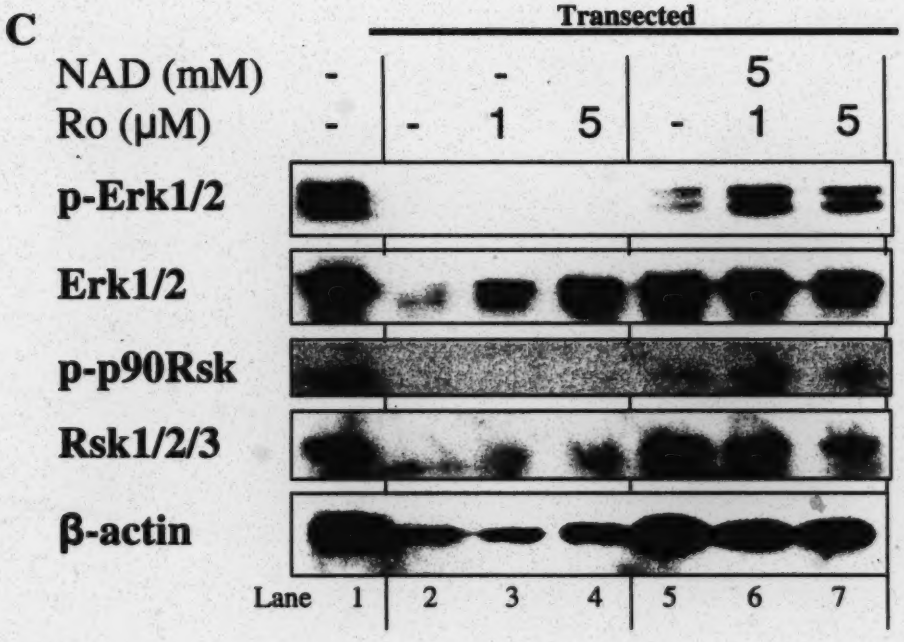


Figure 3.5. Ro-31-8220 increases metabolic activity of NAD-treated, transected axons and increases Erk1/2 phosphorylation

Figure 3.6. Sirtinol increases metabolic activity of NAD-treated, transected axons and is MEK kinase dependent. Distal axons given 50ng/ml NGF were treated with NAD alone, NAD and sirtinol, NAD, sirtinol and U0126, sirtinol and U0126, or sirtinol alone. Control culture distal axons were also given 50ng/ml NGF and left untreated before and after cell body/proximal axon removal or left intact and untreated.

A.– Bright field images of axons stained with MTT 24 hours after cell bodies and proximal axons have been removed. The scale bar is 200μm.

B.– Quantification of MTT stained axons 24 hours after cell body and proximal axon removal. N=7, Data compiled from 2 experiments each with 3-4 cultures per group. Values are presented as the mean absorbance ± SEM. Statistically significant differences between transected controls and treatment groups are indicated by two (Two-Sample Assuming Unequal Variances, $p<0.01$) or three asterisks ($p<0.001$) and statistically significant differences between transected, NAD-treated and transected, NAD- and sirtinol-treated groups are indicated by three pluses (1mM NAD, $p<0.001$) or three number signs (5mM NAD, $p<0.001$).

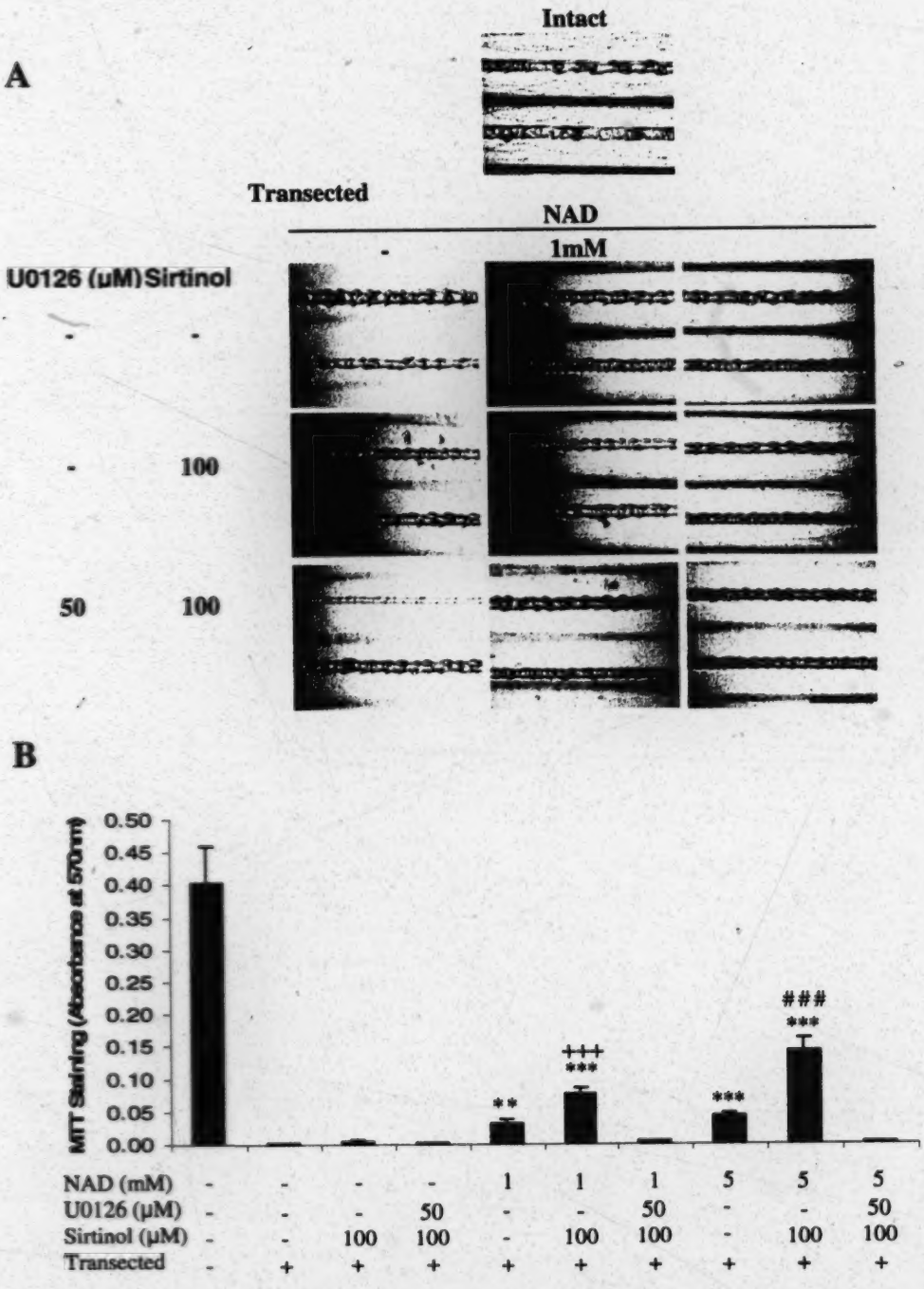


Figure 3.6. Sirtinol increases metabolic activity of NAD-treated, transected axons and is MEK kinase dependent

Figure 3.7. Sirtinol and NAD treatment of transected axons maintains Erk1/2 phosphorylation up to 24 hours. Distal axons given 50ng/ml NGF were treated with combinations of NAD, sirtinol, and U0126, as indicated. Control culture distal axons were also given 50ng/ml and left untreated before and after cell body/proximal axon removal or left intact and untreated. Axons we left treated for 4 hours (A), 8 hours (B), or 24 hours (C) before lysates were prepared for immunoblotting (4 distal compartments per lane). Phosphorylated Erk1/2 (upper panel), total Erk1/2(middle panel), and β-tubulin (lower panel) were visualized with anti-pErk1/2 mAB (1:1000), anti-Erk1/2 pAB (1:10,000) and anti-β-actin mAB (1:1000). Results are representative of 3 experiments.

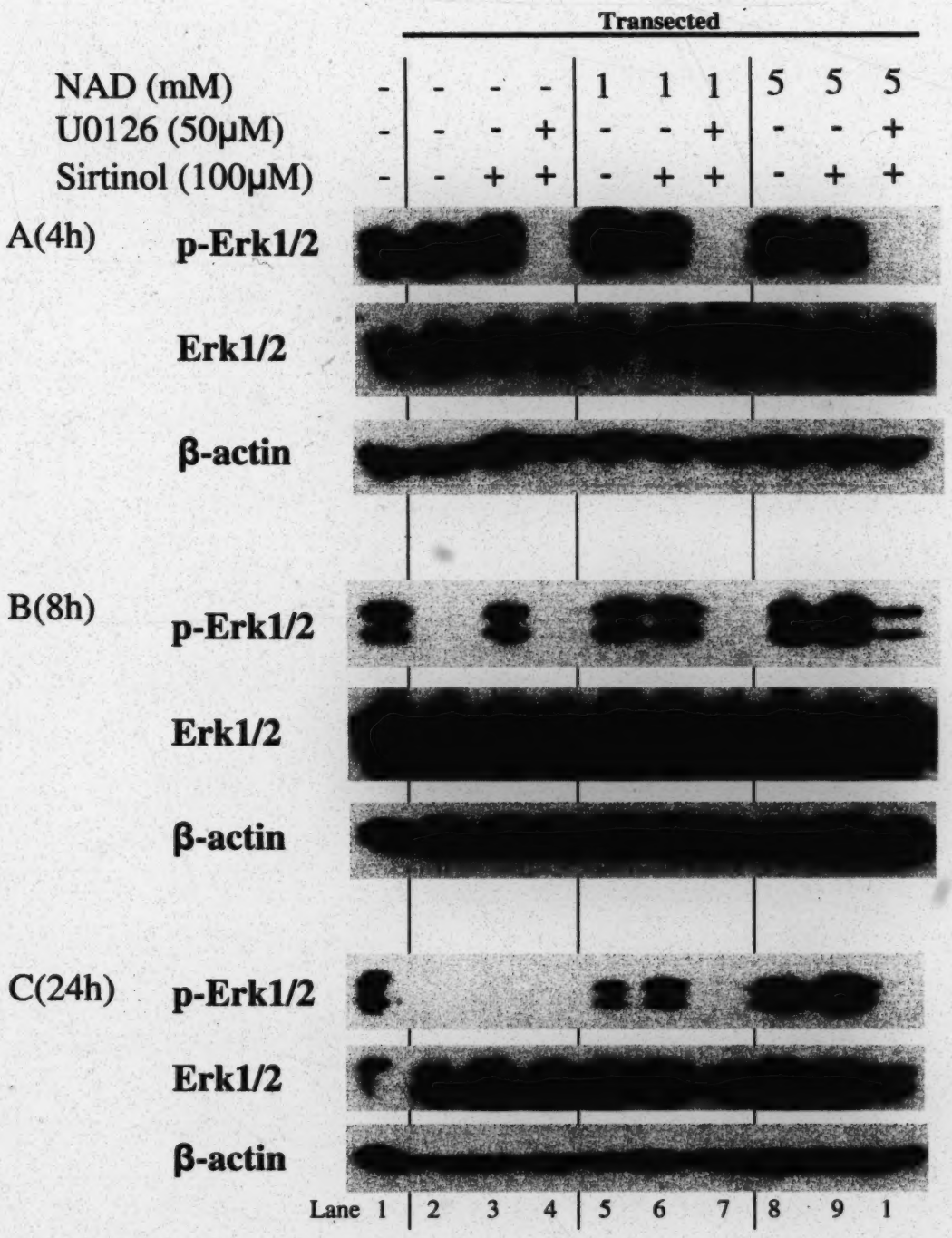


Figure 3.7. Sirtinol and NAD treatment of transected axons maintains Erk1/2 phosphorylation up to 24 hours

4. Discussion

4.1. Delays in Wallerian degeneration of transected axons is mediated by exogenous NAD applied to the axons:

Treatment of axons of sympathetic neurons with exogenous NAD in compartmented cultures, and not treatment of cell bodies, maintained axonal metabolic activity after transection. These results suggested that NAD mediates delays in axonal degeneration not through effects in the cell body as suggested by Araki et al. (Araki et al., 2004), but through direct actions in the axons themselves, as proposed by Wang et al. (Wang et al., 2005). One possible explanation for NAD mediating axonal survival is that treatment of transected axons with exogenous NAD maintains the energetic state of the axon by preventing the decrease in NAD levels reported by Wang et al. (Wang et al., 2005) following transection. NAD functions as a coenzyme in the conversion of glucose to pyruvate. Pyruvate enters the mitochondria where is it further modified into acetyl CoA. Acetyl CoA is then used in the Krebs/TCA Cycle to generate adenosine triphosphate (ATP). Despite this possibility, analysis of mitochondrial activity by the CellTiter 96 One Solution Cell Proliferation Assay (Promega), a derivative of the MTT assay, displayed low metabolic activity in NAD-treated, transected axons.

Before discussing the reduced metabolic effects in NAD-treated, transected axons, some issues with the assay itself should be addressed. The MTT assay was first designed by Mossman (1983) as an assay to assess cellular growth and survival. The use of tetrazolium salt was considered a relatively fast and safe (as it uses no radio isotopes) to assess cellular function through colourimetric changes. The MTT assay uses a tetrazolium salt that is cleaved by active mitochondrial succinate dehydrogenase to form

formazan which results in the formation of blue stain (Mosmann, 1983). Mosmann (Mosmann, 1983) observed the amount of formazan was proportional to the amount of lymphoma cells that were plated, so with more cells present the greater the amount of formazan produced. Given a certain number of cells, the changes in formazan production should indicate cell survival. Many studies use the MTT assay as an indicator of survival and confirmed these results through other survival assays (for example: (Chiou et al., 2006, Hanneken et al., 2006, Schipper et al., 2006)), suggesting the MTT assay is a valid assay for survival. Despite this, the MTT assay only assesses the metabolic function of a cell and cannot be taken as an absolute indicator of survival, especially when cell number is unknown or if metabolic activity is altered. Given this, interpreting the MTT assay as survival assay is complicated by the fact that the exact number of axons in culture is unknown as well as how or if the energetic demands of axons lacking cell bodies are affected. So interpretations about how many axons are alive based on the amount of stain formation are uninformative because nothing is known about axon number or there energetic demands. Despite this, the MTT assay can still be used to indicate axon cables are alive because they are still metabolically active, and using this measure, inferences can be made about NADs ability to delay Wallerian degeneration in transected axons.

As stated above, transected axons displayed low levels of mitochondrial reactivity when treated with NAD compared to axons of intact, untreated neurons (Figure 3.1). A possible explanation for this low level of mitochondrial metabolism could be due to a change in the energy requirements of transected axons. With the removal of the cell bodies, transport of materials from the cell bodies no longer occurs. It is possible that the motor proteins needed for transport of materials from the cell body to the terminal ends

of the axon no longer function, and this could have greatly reduced the energy demand on mitochondria. Although mitochondrial activity appears reduced following 24 hour NAD treatment of transected axons, NAD-treated, transected axons do not appear to be as degenerated as untreated, transected axons (Figure 3.2A). Determining if the energetic demands of transected axons are altered may clarify whether the MTT assay is a poor indicator of transected axon survival.

Delays in Wallerian degeneration resulting from treatment of transected axons with NAD conflict with the report that NAD pretreatment of neuronal cell bodies is what mediates delays in axonal degeneration after transection (Akari et al., 2004). The reason for this difference between studies is unclear, but one possibility is that NAD may mediate protective effects from axon degeneration in both cell body and in the axon *in vivo*. The model system used by Araki et al., (2004) may favour cell body-mediated protection of axon degeneration, while our model system may favour local protection of axons from degeneration. This requires further study to determine whether NAD mediates axonal protection in both the cell body and the axon and if so, how this is achieved.

4.2. The MEK/Erk pathway may be a general signaling pathway necessary for survival in transected axons:

NAD applied to transected axons maintained levels of Erk1/2 phosphorylation and protected the axons from Wallerian degeneration. This protection was abrogated with the application of a MEK inhibitor, U0126, and Erk1/2 phosphorylation was inhibited. Degeneration of transected axons was also restored with MEK inhibition of

transected axons treated with a proteasome inhibitor, MG132, but MEK inhibition has no effect on survival of axons of intact neurons (MacInnis and Campenot, 2005). This suggests that Erk1/2 activity is a general signaling pathway that becomes necessary for the survival of transected axons, but Erk1/2 activity is not necessary for the survival of axons of intact neurons. One possible explanation for Erk1/2 activity being necessary in transected, but not intact, axons is that in intact axons other signaling pathways may compensate for the loss of Erk1/2 activity. It is also possible that Erk1/2 is a redundant pathway in intact neurons that becomes necessary as normal dominant survival pathways are no longer functional in transected axons. Erk1/2 activity has been extensively implicated in studies of neuronal survival following insult by hypoxia, glutamate toxicity and DNA damage (Zhu et al., 2002, Ferchmin et al., 2003, Arai et al., 2004), and this work further demonstrates the importance of Erk1/2 in survival of transected axons.

One possible way Erk1/2 activity may promote survival in transected axons is through preserving mitochondrial function by activating p90Rsk. p90Rsk inactivates the pro-apoptotic protein BAD by sequestering it in the cytosol, preventing its translocation to the mitochondria where BAD causes the release of cytochrome c and induces death (Bonni et al., 1999, Masters et al., 2001). The p90Rsk inhibitor, Ro-31-8220 was used to explore this possibility in NAD-mediated protection of transected axons. Surprisingly, Ro-31-8220 did not inhibit p90Rsk phosphorylation as described previously in the literature (Alessi, 1997, Arai et al., 2004, Nguyen et al., 2005) as an increase in p90Rsk phosphorylation was observed in Ro-31-8220 and NAD treatment compared to NAD treatment alone. Ro-31-8220 was originally described as a PKC inhibitor (Davis et al., 1992) in rat brain homogenates, but has also been shown to activate the pro-apoptotic

proteins glycogen synthase kinase-3 (GSK3) in rat adipocytes and L6 myotubes (Standaert et al., 1999), c-Jun N-terminal kinase (JNK) in rat adipocytes (Beltman et al., 1996, Standaert et al., 1999) and inhibit the activity of mitogen and stress-activated protein kinases 1/2 (MSK1/2) in RAW 264.7 macrophages (Mu et al., 2005). Interestingly, inhibition of MSK1/2 or activation of GSK3 and JNK typically results in the death of the cell and does not help to explain why, along with increases in p90Rsk phosphorylation, improved Erk1/2 phosphorylation and MTT reactivity were observed (Figure 3.5). These results suggest that Ro-31-8220 may have other effects in transected axons that result in increased metabolic function of NAD-treated transected axons and do not inhibit p90Rsk phosphorylation. It is uncertain with the MTT assay alone to determine if NAD and Ro-31-8220 treatment of transected axons are able to improve axon survival.

One possible explanation for this increase in metabolic activity by Ro-31-8220 was presented by Hers et al. (1999). Application of Ro-31-8220 to rat adipocytes demonstrated that Ro-31-8220 did not activate GSK-3, as Standaert et al., (1999) suggest, but in fact reduced its function by 87% compared to controls (Hers et al., 1999). Phosphorylation of GSK-3 by upstream effectors results in its deactivation, and pharmacological inhibition of GSK-3 activity in cerebellar granule neurons following potassium withdrawal protects neurons from apoptosis (Cross et al., 2001). GSK-3 induces apoptosis by destabilizing the bcl-2 family member, myeloid cell leukemia sequence 1 (MCL-1) (Maurer et al., 2006). Destabilization of MCL-1 leads to a loss mitochondrial membrane potential and cytochrome c release, which results in apoptosis. If this is the case, GSK-3 inhibition by Ro-31-8220 may account for the increased

metabolic activity seen with combined treatment of NAD and Ro-31-8220, as the drug may prevent the activation of GSK-3 and its downstream effects on the mitochondria altering metabolic function in combination with the NAD-mediated protection.

GSK-3 is also a downstream target of Erk1/2 activity. Activation of Erk1/2 works to inhibit the activation of GSK-3 and prevent its apoptotic effects in cortical neurons (Hetman et al., 2002). This raises the question of whether Erk1/2 activity inhibits activation of GSK-3 and prevents the loss of mitochondrial membrane potential, maintaining mitochondrial function. GSK-3 may be a possible downstream target of Erk1/2 activity in transected axons, as when Erk1/2 phosphorylation is inhibited GSK-3 activated and destabilizes the mitochondria, restoring normal axon degeneration. Level of GSK-3 phosphorylation could be assessed by western blot with phospho-specific antibodies to address this question. Further investigation of Erk1/2 effects on GSK-3 may potentially lead to new insights for therapeutic treatments of Alzheimer's disease, in which increases in GSK-3 activity have been documented (Gomez-Ramos et al., 2006).

4.3. Treatment of transected axons with sirtinol increases metabolic function of NAD-treated axons, but survival is still dependent on MEK kinase activity:

Metabolic activity of NAD-treated, transected axons was increased with treatment of the SIRT inhibitor sirtinol, but axons degenerated normally with sirtinol treatment alone (Figure 3.6). One possibility for this phenomenon is that sirtinol may have additional effects on transected axons, beyond inhibiting SIRT proteins. Previous results have demonstrated transected axons loose Erk1/2 phosphorylation after 8 hours, but treatment of transected axons with sirtinol alone was able to maintain Erk1/2

phosphorylation for this time period, but not a full 24 hours. SIRT inhibition may cause a delay in the decrease of NAD levels by preventing SIRT-dependent consumption of NAD in the axon. This inhibition may reduce the number of sources responsible for the decrease in amount of NAD in the axon, decreasing the rate at which NAD is lost. This may increase the available NAD present in the NAD- and sirtinol-treated, transected axon compared to the NAD alone treated, transected axon, altering the timeline in which the axon degenerates. A rise in NAD concentration may then facilitate an increase in metabolic function and may delay axon degeneration (Wang et al., 2005). This delay in axon degeneration would result in a more functional state for the transected axon and may explain the higher levels of Erk1/2 phosphorylation seen with NAD and sirtinol treatment. However, this is an unlikely explanation as application of 10 or 20mM NAD to transected distal axons did not result in increased protection of transected axons, which would be expected if more NAD was made available.

Despite the improved metabolic activity with NAD and sirtinol treatment, NAD-mediated protection, with or without sirtinol, is still dependent on MEK/Erk activity, as MEK inhibition abrogates the protective effects of NAD, whether applied alone or combined with sirtinol (Figure 3.7). These combined drug treatments highlight that regardless of how metabolic activity is altered, MEK activity is necessary for survival of transected axons. Application of multiple compounds to transected axons most likely affects multiple targets in those axons. Regardless of how transected axons are treated and survival is maintained or metabolic activity is altered, MEK/Erk activity is necessary for survival as inhibition of this signal transduction pathway resulted in normal axon degeneration.

4.4. Implications of the MEK/Erk pathway in neuronal trauma and disease:

The view of the MEK/Erk pathway in neuronal survival is currently changing from the pathway being involved in the death of a neuron to being involved in its survival (reviewed by Chu et al., 2004; Hetman and Gozdz, 2004). Studies using hypoxia, transection, glutamate toxicity and trophic factor withdrawal suggest Erk1/2 activity becomes an important pathway in survival only after the neuron has undergone some sort of damaging insult. This study further suggests that Erk1/2 activity is a necessary pathway for axonal survival following transection. Mechanisms of axon degeneration have been shown to be independent of the cell body with the discovery of the Wlds mutant, and axonal degeneration appears not to be the result loss of the supply of biosynthetic material from the cell body. Instead this suggests that regulatory mechanisms in Wlds mice are somehow altered to delay Wallerien degeneration and the MEK/Erk signaling pathway may be involved in regulating these mechanisms of degeneration. Also, dying-back axon degeneration and Wallerian degeneration may share similar mechanisms of axon degeneration and if this is the case, Erk activity is an attractive target for therapeutic treatment for a variety of neuronal diseases. Since symptoms in diseases such as ALS or Alzheimer's disease arise from the loss of axon connectivity, treatments that improve Erk1/2 signaling may be able to delay symptom development in afflicted individuals. A better understanding of the downstream targets in the MEK/Erk pathway involved in axonal survival may provide new insights in protection of damaged axons and lead to better therapeutic treatments of neurodegenerative diseases.

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